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TOXICITY OF POLYCHLORINATED DIPHENYL ETHERS IN *HYDRA ATTENUATA* AND IN RAT WHOLE EMBRYO CULTURE

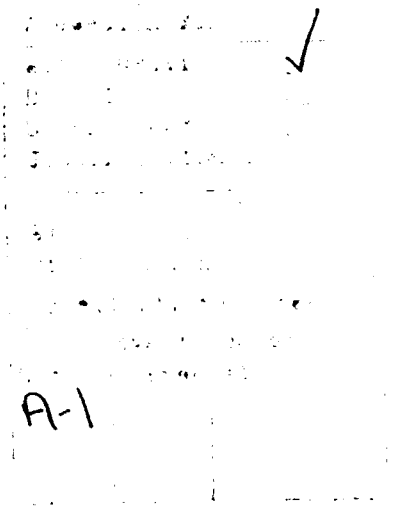
A Thesis

by

MARION CAROL BECKER

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE



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Major Subject: Toxicology

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May 1991

ABSTRACT

Toxicity of Polychlorinated Diphenyl Ethers in *Hydra Attentuata*

and in Rat Whole Embryo Culture. (May 1991)

Marion Carol Becker, B.S., University of Arkansas;

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Co-Chairs of Advisory Committee: Dr. Stephen H. Safe
Dr. Timothy D. Phillips

Polychlorinated diphenyl ethers (PCDEs) are a class of biaryl compounds that have little commercial application, but appear to be widespread in the environment. They have been found in wood preservative waste dumpsites and in fly ash from municipal waste incinerators. They have been detected in bird eggs and tissues, fish, and other edible marine organisms in the United States, Canada, and Europe. There are limited reports in the extant literature on the toxicity of PCDEs. This study was designed to evaluate the toxicity of selected PCDEs in cultures of *Hydra attenuata* and post-implantation rat whole embryos. The toxicity of several closely related polychlorinated biphenyls (PCBs) was evaluated in both cultures and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) was evaluated in whole embryo culture. Adult hydra were exposed to 3,3′,4,4′-tetrachlorodiphenyl ether, 2,3′,4,4′,5-pentachlorodiphenyl ether, 3,3′,4,4′,5-pentachlorodiphenyl ether, 2,3,3′,4,4′,5-hexachlorodiphenyl ether, 2,2′,4,4′,5,5′-hexachlorodiphenyl ether, 2,2′,3,3′,4,4′,5,6,6′-nonachlorodiphenyl ether, 2,3′,4,4′,5-pentachlorobiphenyl, or

3,3′,4,4′,5-pentachlorodiphenyl (dissolved in DMSO) for 92 hours. In no case was the toxic endpoint reached in the adult hydra with test chemicals at the maximum soluble concentrations (≤ 100 μg/ml). Extracorporeally maintained rat embryos were cultured in rat serum containing test chemicals (≤ 250 μg/ml) for 45 hours. Embryos were exposed to 3,3′,4,4′-tetrachlorodiphenyl ether, 3,3′,4,4′,5-pentachlorodiphenyl ether, 2,2′,4,4′,5,5′-hexachlorodiphenyl ether, 2,3,3′,4,4′,5-hexachlorodiphenyl ether, 3,3′,4,4′-tetrachlorobiphenyl, or 2,3,7,8-TCDD. Embryonic growth and development parameters (yolk sac diameter, crown-rump length, somite count, and DNA and protein content) and gross morphology were determined. Findings indicated that these chemicals were neither embryotoxic nor teratogenic. Thus, the PCDEs, which elicit other diverse toxic and biochemical responses in rodents, are relatively inactive in these bioassays for developmental toxicity.

DEDICATION

I dedicate this thesis to Dr. and Mrs. Ritchey. Their friendship, kindness, love, and support have helped me more than words can ever express.

ACKNOWLEDGEMENTS

I wish to express my deep appreciation to Dr. Timothy D. Phillips who provided not only the laboratory space, but also the ideas and encouragement to bring this study to fruition. Sincere thanks to Dr. Stephen H. Safe for his guidance, inspiration, and wonderful sense of humor, and to Dr. Leon F. Kubena for serving on my thesis committee.

I would like to extend a special word of appreciation to Dr. Kittane Mayura for her assistance in the laboratory. Special thanks to Dr. Beverly A. Clement whose advice I frequently sought, to Mrs. Modene Botter for her ability to supply me with materials even on short notice, and to Ms. Carrie Nichols for assisting me with the final preparation of this manuscript. I would like to express special words of thanks and appreciation to Dr. James H. Wright for the hours he spent proof-reading this manuscript, and for his invaluable comments and suggestions.

I am indebted to the United States Air Force for providing me with the opportunity to devote two years to study toxicology along with the financial support to do so. I especially appreciate all of my friends and fellow officers in the United States Air Force who have given me moral support as well.

Finally, I would like to extend a very special word of thanks to Cathy Dryden and all my colleagues and friends in the Department of Veterinary Pharmacology and Physiology and the Department of Veterinary Anatomy and Public Health. Their encouragement, camaraderie, and support kept me working hard--often times against my

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CHAPTER I

INTRODUCTION

The halogenated dibenzo-*p*-dioxins (dixons), dibenzofurans (furans), biphenyls, naphthalenes, and terphenyls comprise a group of structurally related compounds of considerable environmental interest because of their ubiquity, stability, and potential for biomagnification and toxicity (Morrissey and Schwetz, 1989). Another group of compounds, the polychlorinated diphenyl ethers (PCDEs), has recently been added to this list. Unlike the dioxins, furans, and biphenyls, little is known about the toxicity of PCDEs.

PCDEs are widely distributed and are known contaminants in the commonly used chlorophenol preparations (Villaneuva *et al.*, 1973; Nilsson and Renberg, 1974; Nilsson *et al.*, 1978; Paasivirta *et al.*, 1982). They have been detected in edible marine organisms in Narragansett Bay, RI (Lake *et al.*, 1981), in avian eggs and tissue in various locations in the United States and Canada (Stafford, 1983), and in fresh salmon, commercial fish liver oil, and in white-tailed eagles in Finland (Paasivirta *et al.*, 1986). Stanley *et al.* (1990), tested samples of composited human adipose tissues for PCDE contamination. All samples were positive for PCDEs with concentrations as high as 2000 ppt.

Initial studies indicate that the PCDEs have an uptake and distribution pattern similar to that of polychlorinated biphenyls (PCBs) (Zitko and Carson, 1977; Newsome *et al.*, 1983). Their metabolic pathways, on the other hand, differ from PCB pathways in

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This proposal follows the style of the Journal of *Toxicology and Applied Pharmacology*

that all PCDEs investigated by Tulp *et al.* (1979), gave rise to *ortho*-hydroxylated metabolites whereas with PCBs, *ortho*-hydroxylated metabolites are rarely observed.

The PCDEs exhibit varying physicochemical properties. Some have been classified as 3-methylcholanthrene-type, some as phenobarbital-type, and others as mixed-type inducers of xenobiotic metabolism (Chui *et al.*, 1985; Iverson *et al.*, 1987). Iverson *et al.* (1987), tested 12 PCDEs for monooxygenase induction properties in rats and concluded that the structure-activity rules formulated by Safe *et al.* (1985), for PCBs also apply to these 12 PCDEs. However, subsequent studies with C57BL/6N mice (Howie *et al.*, 1990) have shown that these rules do not apply to all PCDEs.

Studies on rats given oral doses of selected PCDEs indicate that the liver and thyroid are target organs for these ethers. Morphological changes in these organs were mild, however, and investigators have concluded that these ethers are only moderately toxic in the rat (Chu *et al.*, 1989, 1990). To date, the teratogenic potential of PCDEs has not been investigated and little is known about their potential for causing developmental defects.

Because the PCDEs are closely related to the chlorinated dibenzo-*p*-dioxins and the PCBs, it is of interest to review studies conducted to determine the developmental toxicity of some of these related compounds. Studies have shown that 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD) is a potent inducer of cleft palate and hydronephrosis in mice (Poland and Glover, 1980). Differences in sensitivity of mice to 2,3,7,8-TCDD have long been recognized. Poland and Glover (1980) reported that in 9 out of 10 inbred strains studied, the susceptibility to cleft palate formation produced by 2,3,7,8-TCDD followed

the strain distribution of the aryl hydrocarbon (Ah) locus. In whole embryo culture in the rat, no abnormal development was seen at concentrations up to 90 ng/ml 2,3,7,8-TCDD. Although development was normal at this concentration, the study confirmed that 2,3,7,8-TCDD did, in fact, reach the embryo (Neubert *et al.*, 1987).

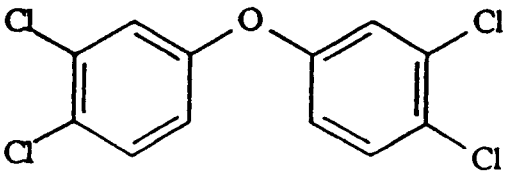
The teratogenic effects of th^XxPCBs have also been demonstrated. They are known to cross the placenta in several species, including humans (Kimbrough *et al.*, 1978). In albino mice, the predominant malformations detected with 3,3´,4,4´,5,5´-hexachlorobiphenyl were cleft palate and hydronephrosis (Marks *et al.*, 1981). Oral administration of 3,3´,4,4´-tetrachlorobiphenyl to Sprague-Dawley rats caused accumulation of blood in the amniotic fluid, and in the intestines and the stomachs of fetuses. This PCB was embryolethal at some doses (Wardell *et al.*, 1982).

It is not feasible to conduct exhaustive teratogenic testing on every chemical to which women of child-bearing age may be exposed. For this reason, experiments in developmental toxicology should focus on identifying those compounds which are capable of injuring the conceptus at a concentration well below that which is toxic to the mother. Standard developmental toxicity safety testing must be designed to distinguish between substances uniquely hazardous to the conceptus and those which are coaffective agents, affecting both mother and fetus (Johnson and Christian, 1985).

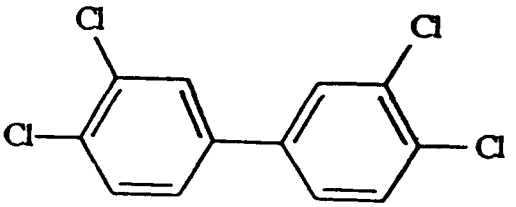
Currently, chemicals are tested for reproductive toxicity in whole animals during 3 phases of exposure: 1) before mating to weaning; 2) during organogenesis; and 3) during the perinatal period (Sadler *et al.*, 1982). Using such a 3 tier protocol, it would be virtually impossible to test every chemical already in the inventory, not to mention the new

chemicals being introduced each day. Alternative screening procedures capable of predicting which of these compounds could have adverse effects on the developing mammalian embryo are essential. These screening tests should produce rapid results, be inexpensive, reproducible, and simple to perform. Both the hydra assay and post-implantation whole embryo culture can be useful systems for teratogenic screening of chemicals.

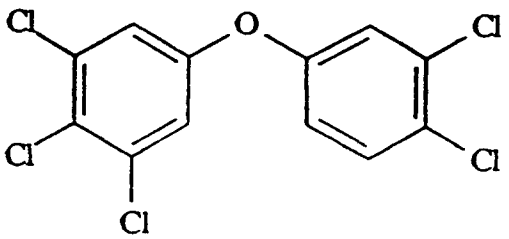
The first objective of this study was to test selected PCDEs for teratogenicity using the *Hydra attenuata* system, a screening test for the rapid detection and prioritization of developmental toxins (Johnson *et al.*, 1982). The second objective was to confirm the findings of the hydra assay by testing the same PCDEs via another *in vitro* assay, the rat whole embryo culture developed by New (1978). The third objective was to compare the potential for developmental toxicity between selected PCDEs and their corresponding PCBs, some of which are known to be teratogenic in mice. Fig. 1 outlines the structures of test compounds evaluated in this study.



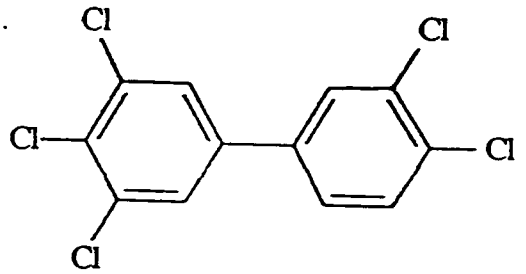
3,3′,4,4′-Tetrachlorodiphenyl ether



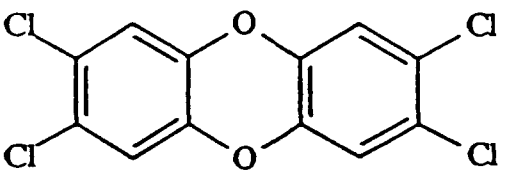
3,3′,4,4′ Tetrachlorobiphenyl



3,3′,4,4′,5-Pentachlorodiphenyl ether

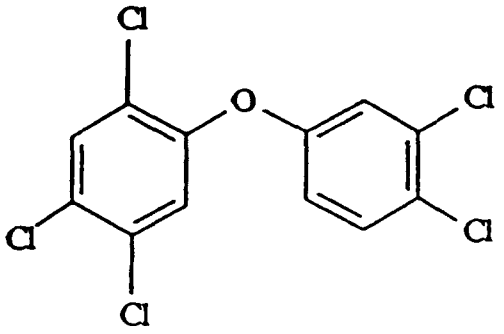


3,3′,4,4′,5-Pentachlorobiphenyl

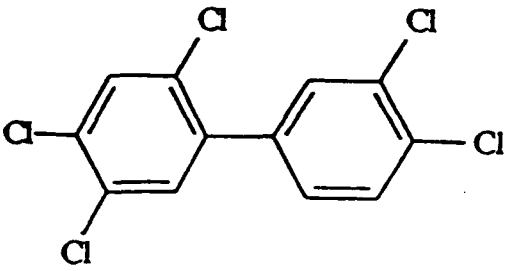


2,3,7,8-Dibenzo-*p*-dioxin

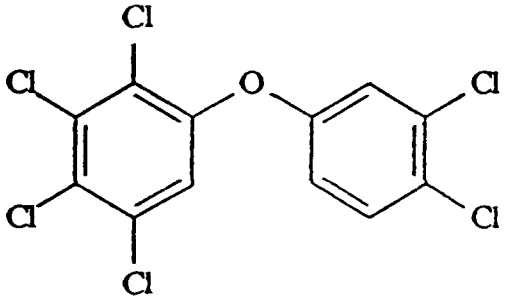
FIG. 1. Structures of polychlorinated diphenyl ethers, polychlorinated biphenyls, and 2,3,7,8-TCDD used in this study.



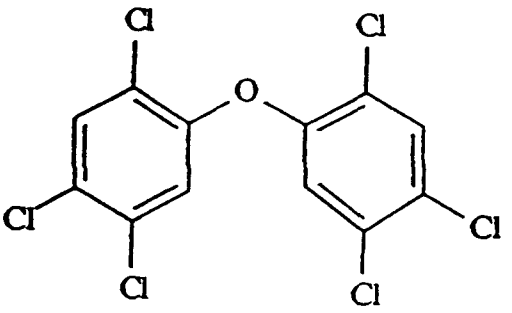
2,3′,4,4′,5-Pentachlorodiphenyl ether



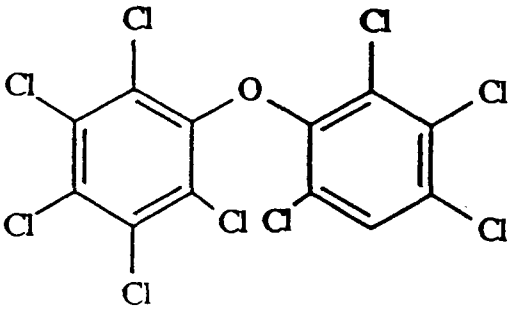
2,3′,4,4′,5-Pentachlorobiphenyl



2,3,3′,4,4′,5-Hexachlorodiphenyl ether



2,2′,4,4′,5,5′-Hexachlorodiphenyl ether



2,2′,3,3′,4,4′,5,6,6′-Nonachlorodiphenyl ether

FIG. 1. Continued

CHAPTER II

LITERATURE REVIEW

Detection in the Environment. PCDEs are a class of biaryl ethers that contain from 1 to 10 chlorine substituents and altogether comprise a family of 209 congeners and isomers. These ethers have physical and chemical properties very similar to those of PCBs (Tulp *et. al.*, 1979).

PCDEs have not been used extensively on an industrial or commercial level, but have had limited use as hydraulic fluids and as thermostable heat exchange liquids (Rappe *et al.*, 1979). Despite their limited commercial application, they are well recognized environmental contaminants (Lake *et al.*, 1981; Stafford, 1983; Iverson *et al.*, 1986; Paasivirta *et al.*, 1986; Williams and LeBel, 1988; Chu *et al.*, 1989, 1990).

They have been detected, along with polychlorinated dibenzofurans (PCDFs), in samples of edible marine organisms and in suspended particulate matter from Narragansett Bay, RI. Tissue samples taken from mussels, clams, and lobsters in that region were all positive for PCDEs and PCDFs. Narragansett Bay is used as a commercial fishery and, at the same time, a disposal site for industrial and municipal wastes. The pollutants detected in Narragansett Bay most likely originated from discharges of industrial chemical plants in the northern bay area (Lake *et al.*, 1981). The extent of PCDE and PCDF contamination in other estuaries is unknown.

PCDEs have also been detected in samples of avian eggs and tissue taken from birds in Louisiana, Michigan, Ohio, Rhode Island, Texas, and Ontario, Canada. The

highest concentration quantified was 0.90 ppm of an unidentified tetrachlorodiphenyl ether isomer in the carcass of a common tern (Stafford, 1983). Studies conducted in Finland have detected PCDEs in fresh salmon, white-tailed eagles, and in commercial fish liver oil (Paasivirta *et al.*, 1986). Fly ash from municipal waste incinerators in Helsinki, Finland, was positive for hexa- and heptachlorodiphenyl ethers (Paasivirta *et al.*, 1986). Because few studies have been conducted to determine the toxicity of these ethers, the significance of these residues in the environment is unknown.

Chemical treatment of sawmill wood waste products produces various degradation products, depending upon the chemical treatment method used. When waste products are treated with NaOCl and NaOH the content of chlorophenols and phenolic dimers in waste products can be lower than pretreatment levels. However, treatment with NaOCl can increase the content of neutral dimers such as PCDEs, PCDFs, and polychlorinated dibenzo-*p*-dioxins (PCDDs). Treatment with NaOH, on the other hand, does not change the composition (Paasivirta *et al.*, 1982).

PCDEs have been shown to be relatively stable in the environment (Garå *et al.*, 1981). This, coupled with the fact that they are physicochemically similar to the PCBs, suggests that leakage of PCDEs into the biosphere may cause bioaccumulation problems similar to those caused by PCBs (Sundström and Hutzinger, 1976). Furthermore, studies indicate that the rate of degradation of PCDEs depends upon their concentration. As the concentration decreases, PCDEs take much longer to degrade (Norström and Andersson, 1977). Consequently, PCDEs will be found as environmental contaminants for many years.

Found as Impurities in Chlorophenols. The widespread appearance of PCDEs in the environment is most likely due to their presence as impurities in chlorophenol preparations. Infrared spectrophotometry and mass spectrometry have confirmed the presence of PCDDs, PCDFs, and PCDEs in samples of technical and analytical grade, commercially available chlorophenols (Villaneuva *et al.*, 1973; Nilsson and Renberg, 1974; Nilsson *et al.*, 1978; Paasivirta *et al.*, 1982). According to Stafford (1983), PCDEs containing from 3 to 10 chlorines have been identified in various chlorophenol preparations. Some of these halogenated contaminants are quite likely more toxic than the chlorophenols themselves (Vos *et al.*, 1970; Woolson *et al.*, 1972).

Commercial chlorophenol preparations are widely used as wood preservatives, bacteriostats, fungicides, slimicides, and as key intermediates in the production of chlorinated phenoxyacetic acids. Chlorinated phenoxyacetic acids are widely used as herbicides (Rappe *et al.*, 1979; Stafford, 1983). Nilsson and Renberg (1974), using mass spectrometric fragmentation, estimated levels of PCDEs in commercial chlorophenols to be in the range of 100 to 1000 ppm. There is considerable variation in concentration since by-products of the production of chlorophenol formulations vary greatly depending upon the production method used.

Conversion to More Toxic Compounds. Although few studies have been conducted to determine the toxicity of PCDEs, these ethers are possible precursors to the highly toxic PCDFs and PCDDs, both in industrial processes and in natural photolytic reactions in the environment (Norström *et al.*, 1976a, 1976b; Choudhry *et al.*, 1977; Lindahl *et al.*, 1980). The toxicity of PCDFs and PCDDs has been extensively

documented (Goldstein and Safe, 1989; Morrissey and Schwetz, 1989). Hence, the conversion of PCDEs into PCDFs and PCDDs can be environmentally significant.

Though open fire burning of chlorophenol formulations removed all PCDEs from the samples, PCDF concentrations increased slightly and PCDD concentrations increased even more compared to precombustion levels in the formulations (Paasivirta *et al.*, 1982). Rappe *et al.* (1979), showed that thermal conversion of PCDEs into PCDFs is of the same order as conversion from PCBs.

Photochemical breakdown of PCDEs can follow 2 competitive pathways. They can either undergo dechlorination or ring closure whereby chlorinated dibenzofurans are formed (Norström *et al.*, 1976a). For this photolytic ring closure to occur, there must be at least one *ortho* chlorine in the PCDE. When PCDEs were heated in sealed quartz ampules, PCDFs were formed primarily through loss of *ortho* HCl and *ortho* H_2. In one experiment, PCDFs were formed as a result of the loss of *ortho* Cl_2 (Lindahl *et al.*, 1980) (Fig 2).

Conversion of PCDEs to the highly toxic PCDFs has been carried out in the laboratory with a one-step synthesis using a palladium (II) acetate catalyzed ring closure (Norström *et al.*, 1976b). The mechanism for this reaction is postulated to involve nucleophilic attack by palladium on both *ortho* carbons with the subsequent loss of an *ortho* chlorine (Norström *et al.*, 1976b).

Since separation procedures are confounded by the fact that PCDEs interfere with the determination of PCDFs by mass spectrometry, conflicting data has been reported regarding the transformation of PCDEs into more toxic substances. During mass

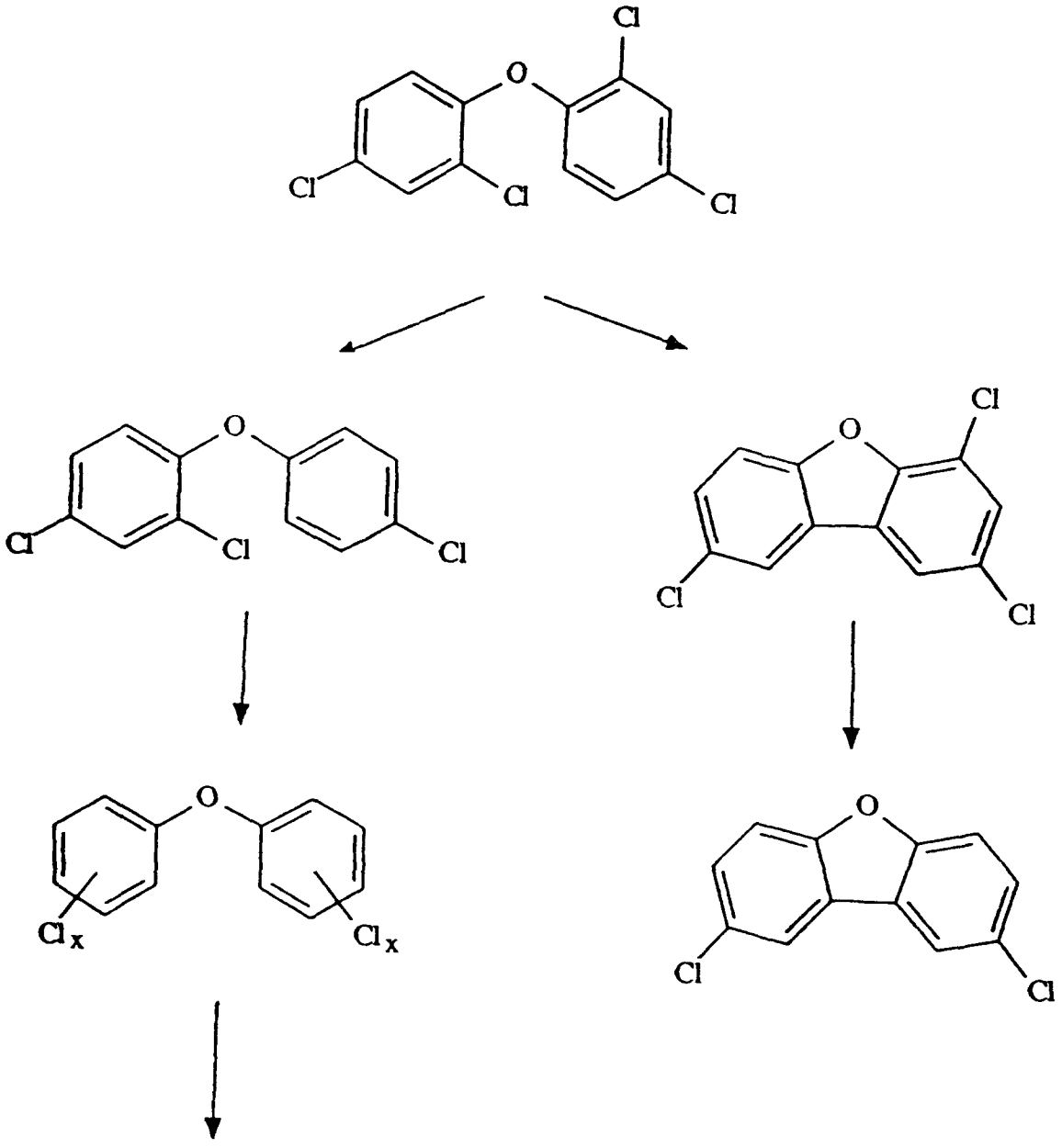


FIG. 2. Photochemical conversion of polychlorinated diphenyl ethers into polychlorinated dibenzofurans.

spectrometry, the PCDEs fragment by loss of 2 chlorines to give the exact mass as a furan of 2 chlorines less. Thus, any analytical technique for the measurement of dioxins and furans must distinguish them from PCDEs. All of these compounds are commonly found in chlorophenol preparations. Although the PCDEs have extraction, high-performance liquid chromatographic, and gas chromatographic properties similar to those of the furans, PCDEs are well resolved from the furans on Florisil columns using hexane and dichloromethane as eluting solvents (Ryan *et al.*, 1984).

Metabolism. Tulp *et al.* (1979), investigated the principle metabolic routes of 4,4′-dichlorodiphenyl ether, 2′,3,4-trichlorodiphenyl ether, 2,4,4′,5-tetrachlorodiphenyl ether, and 3,3′,4,4′-tetrachlorodiphenyl ether in rats. All PCDEs gave rise to *ortho*-hydroxylated metabolites. If the parent compounds had a chlorine in the *ortho* position relative to the ether bond, *ortho*-hydroxylation could lead to the formation of predioxins. Studies of PCDE metabolism in the rat demonstrated that hydroxylation, most likely at both the 2 and 2′ positions, occurred as the major metabolic pathway and that scission of the ether bond was a minor metabolic route (Tulp *et al.*, 1979) (Fig. 3).

One might expect that since the major metabolic pathway for PCDEs is *ortho*-hydroxylation, metabolic conversion to PCDFs and PCDDs could occur *in vivo*. To date, however, products of metabolic cyclization of PCDEs to PCDFs, PCDDs, or to their hydroxylated derivatives have not been detected in excreta. Since PCDFs and PCDDs are excreted unchanged in feces and as hydroxylated derivatives in urine, the absence of any of these metabolites in excreta could indicate that no conversion has occurred (Tulp *et al.*, 1979).

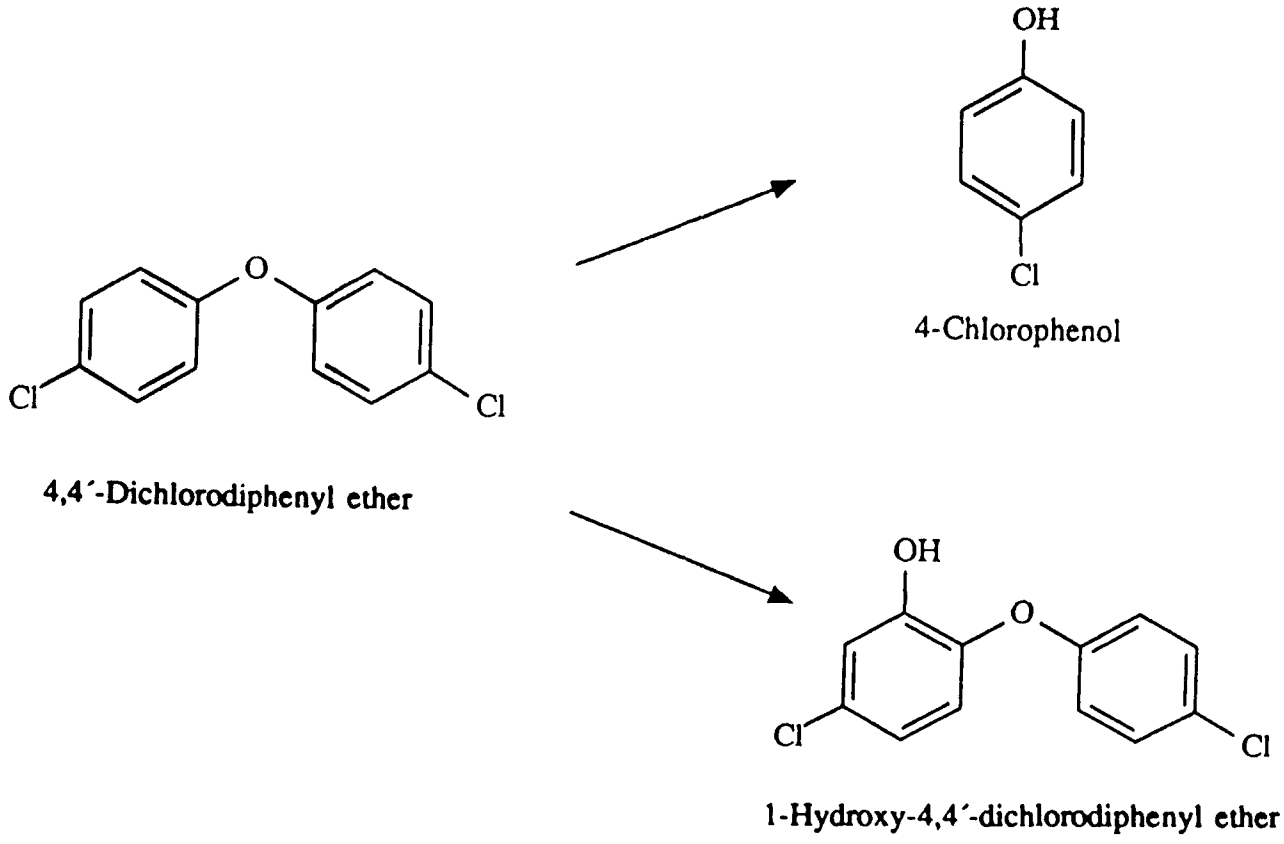


FIG. 3. Metabolic pathways of selected polychlorinated diphenyl ethers.

Similar to PCB metabolism in rats, the metabolic hydroxylation of PCDEs leads to a relatively large number of mono-, di-, and trisubstituted analogues. However, in the case of PCDEs, *ortho*-hydroxy derivatives are major metabolites, whereas in PCBs, they are rarely observed (Tulp *et al.*, 1979).

In trout, 4-chlorodiphenyl ether and 2,4-dichlorodiphenyl ether are hydroxylated primarily at the 4′ position. When the 4 and 4′ positions are occupied as in 2,4,4′-trichlorodiphenyl ether and 2,4,4′,5-tetrachlorodiphenyl ether metabolic rates seem to be slower (Chui *et al.*, 1990).

Uptake, Distribution, and Excretion. Zitko and Carson (1977) studied the uptake, distribution, and excretion of 2,4,4′-trichlorodiphenyl ether, 2,3′,4,4′-tetrachlorodiphenyl ether, and 2,2′,4,4′,5-pentachlorodiphenyl ether in juvenile Atlantic salmon. The uptake and excretion of these ethers resembles that of the corresponding PCBs, although PCDEs appear to be more persistent in the salmon. Half-lives for the 3 chlorodiphenyl ethers tested were 235 hr, 370 hr, and 370 hr, respectively. Accumulation coefficients were concentration dependent and did not show a trend relative to the number of chlorines (Zitko and Carson, 1977).

Results of distribution and excretion studies conducted by Newsome *et al.* (1983), also indicated that the higher chlorinated PCDEs can be expected to show an accumulation and tissue distribution pattern similar to that reported for PCBs. In rats, the PCDE was rapidly transferred to blood, liver, and muscle, reaching peak values after approximately 8 hr. A somewhat slower incorporation was observed with adipose tissue and skin. Highest post-absorptive levels of PCDEs were found in adipose tissue followed

by skin > liver > muscle > blood. Fecal excretion accounted for 20% of the initial dose within the first 7 days, while only 0.04% was eliminated in the urine over this same interval (Newsome *et al.*, 1983). ^{14}C-2,2′,4,4′,5-pentachlorodiphenyl ether followed a similar distribution pattern. Radioactivity was found in all rat tissue examined with the greatest concentration in adipose tissue > skin > liver > kidney > muscle (Komsta *et al.*, 1988).

Chui *et al.* (1990), demonstrated that PCDEs are significantly bioaccumulated by fish from their aqueous environment. Their studies showed that 4-chlorodiphenyl ether and 2,4-dichlorodiphenyl ether are more toxic to trout than 2,4,4′-trichlorodiphenyl ether and 2,4,4′,5-tetrachlorodiphenyl ether. Their data, although limited, suggests that the acute toxicities of the PCDEs are very similar to those of low-chlorinated PCBs. The PCDEs have a very rapid uptake in trout. In contrast, their rate of elimination is slow and seems to be determined by their lipophilicity and biotransformation rates (Chui *et al.*, 1990). It can be expected, as with PCBs, food and environmental samples will retain PCDE residues long after exposure (Newsome *et al.*, 1983).

As part of a program to assess human exposure to toxic chemicals, Canadian scientists examined adipose tissue from cadavers for the presence of PCDEs. The cadavers were from 5 Canadian municipalities. The mean age for males was 63 years and 70 years for females. No PCDE isomers were detected in these tissues at a detection limit of 20 μg/g (Williams and LeBel, 1988).

In a more recent study (Stanley *et al.*, 1990), PCDEs ranging from hexa- to deca-congeners, were detected in composited human adipose tissue samples, with nonachloro-

diphenyl ethers detected in all samples at estimated concentrations as high as 2000 ppt. The composites were prepared to provide human body burden data based on the 9 U.S. census divisions and three age groups (0-14, 15-44, and 45+ years). This study, with its ability to detect low levels of PCDEs, demonstrates the ubiquity of these ethers in human adipose tissue. Identification and quantification of these ethers in human adipose tissue can be a significant factor in determining the sources of exposure to PCDDs and PCDFs (Stanley *et al.*, 1990).

Tissue from chickens raised on wood chip litter that had been exposed to pentachlorophenol was analyzed for PCDE residues. Hepta-, octa-, and nonachloro-substituted congeners predominated in the tissue. These congeners are generally the most prevalent in commercial pentachlorophenol preparations (Newsome and Shields, 1982).

Studies on Sprague-Dawley rats showed that after 7 days, 55% and 1.3% of an orally administered dose of ^{14}C-2,2′,4,4′,5-pentachlorodiphenyl ether was excreted in the feces and urine, respectively. Unchanged pentachlorodiphenyl ether accounted for 64% of the fecal radioactivity, while hydroxylated pentachlorodiphenyl ether accounted for 23%. Among the metabolic products, the monohydroxylated metabolite predominated (Komsta *et al.*, 1988).

Toxicity. Experimental results of PCDE toxicity studies vary considerably depending upon the particular congener under investigation. In general, PCDEs are much poorer inducers of xenobiotic metabolism than might be expected based on studies of polybrominated diphenyl ethers (PBDEs). PCDEs with only 2 or 3 chlorines did not increase O-ethyl O-*p*-nitrophenyl phenylphosphonothionate (EPN) detoxification, or nico-

tinamide adenine dinucleotide phosphate (NADPH) cytochrome *c* reductase, cytochrome P-450, or aryl hydrocarbon hydroxylase (AHH) activity. In general, the higher chlorinated PCDEs were better inducers with decachlorodiphenyl ether causing greatest increases in the induction of these enzyme activities (except for AHH induction) (Carlson *et al.*, 1980).

Chui *et al.* (1985), examined the effects of PCDEs on hepatic mixed-function oxidase (MFO) activities and on liver ultrastructure in trout and rats after ip administration of 100 mg/kg/day of a mono-, di-, tri-, or tetrachlorodiphenyl ether congener for 3 days. Their results indicated that a PCDE with only 3 chlorines (2,4,4´-trichlorodiphenyl ether) can, in fact, induce aminopyrine N-demethylase (ADPM). They classified 2,4,4´-trichlorodiphenyl ether as a phenobarbital-type inducer. These results conflict with the results reported by Carlson, and can possibly be explained by the fact that different pretreatment routes and different exposure concentrations were used in the 2 studies (Chui *et al.*, 1985).

Kerkvliet *et al.* (1985), examined the humoral immunosuppressive effects of several contaminant fractions and purified isomers from technical grade pentachlorophenol. C57BL/6 mice were treated with a single oral dose of the various contaminants 2 days prior to a challenge with sheep erythrocytes (SRBC). The peak splenic IgM antibody response was measured 5 days later. Technical grade pentachlorophenol produced a dose-related suppression of the antibody response while analytical grade pentachlorophenol was without effect. Results indicated that a chlorinated dioxin/furan fraction extracted from the pentachlorophenol was significantly

immunosuppressive, whereas a chlorinated phenoxyphenol fraction and a chlorinated diphenyl ether fraction were inactive when administered at dose levels equivalent to those present in an ID50 dose of technical grade pentachlorophenol (Kerkvliet *et al.*, 1985). The chlorinated diphenyl ether fraction was not immunosuppressive at any dose level tested. The experimental fraction contained 2 hexa-, 5 hepta-, 4 octa-, and 2 nonachlorodiphenyl ether congeners.

Technical grade pentachlorophenol can typically contain 100-1000 ppm levels of PCDEs (Nilsson and Renberg, 1974), and therefore the failure to observe an immunotoxic response was unexpected since it has been assumed that the PCDEs and PCBs exhibit comparable structure/activity relationships (Carlson *et al..*, 1980). In rats, the chlorinated diphenyl ether fraction used in Kerkvliet's study failed to induce any enzyme activity characteristic of the toxic, Ah receptor agonists. This suggested that the active PCDE isomers were not present in that fraction at biologically significant levels (Kerkvliet *et al.*, 1985). This PCDE fraction was also administered to mice at a dose level in the range of the maximum expected level of the ID50 of technical pentachlorophenol and also at several-fold higher doses. Again, the PCDE fraction was not immunotoxic (Kerkvliet *et al.*, 1985).

PCDEs and PCBs exhibit similar activities as inducers of hepatic mixed function oxidases (MFOs) in trout and rats. Chui *et al.* (1985), classified PCDEs as either phenobarbital-type or mixed-type inducers. In later studies, Iverson *et al.* (1987), suggest that PCDEs produce toxic responses at the cellular level by binding to the Ah receptor and this also results in the parallel induction of the forms of cytochrome P-450 that are

increased by 3-methylcholanthrene (3-MC) rather than those induced by phenobarbital. This 3-MC-like activity is postulated to result from the near coplanarity of the 2 phenyl rings which occurs where there are no bulky *ortho* substituents. Additionally, 3-MC induction by PCBs and PCDEs is enhanced in compounds which contain substituents on both *para* and at least one *meta* position. As *ortho* substituents are added, phenobarbital-like activity predominates (Iverson *et al.*, 1987). This relationship was shown to be true for PCB congeners (Safe *et al.*, 1984). According to results reported by Iverson *et al.* (1987), this structure-induction relationship was also apparent for PCDE congeners.

Iverson *et al.* (1987), tested 12 PCDE congeners for their ability to induce liver cytochrome P-450 and related monooxygenase enzyme activities in Sprague-Dawley rats. In their study, all compounds increased P-450 levels or increased monooxygenase enzyme activities in a manner resembling the induction observed after treatment with 3-MC, phenobarbital, or with their combination (PB and 3-MC). Phenobarbital increased APDM activity but did not increase ethoxyresorufin O-deethylase (EROD) activity. In contrast, 3-MC showed no increase in APDM activity but significantly increased EROD activity. These responses were similar to those of the PBDEs, some of which are known to be toxic (Iverson *et al.*, 1987).

Chu *et al.* (1989), administered oral doses (0.04, 0.4, 4.0, or 40 mg/kg/day) of 2,2′,4,4′,6-pentachlorodiphenyl ether, 2,2′,4,4′,5,6′-hexachlorodiphenyl ether, or 2,2′,3,3′, 4,6′-hexachlorodiphenyl ether to male and female rats for 28 days. Treatment with these 3 congeners resulted in no suppression of growth rate or food consumption. Increased liver weights were seen in animals of both sexes fed 40 mg/kg 2,2′,4,4′,5,6′-

hexachlorodiphenyl ether, in males treated with 40 mg/kg 2,2′,4,4′,6-pentachlorodiphenyl ether, and in females fed 40 mg/kg 2,2′3,3′4,6′-hexachlorodiphenyl ether. Treatment with 40 mg/kg of 2,2′,4,4′,5,6′-hexachlorodiphenyl ether significantly increased hepatic microsomal APDM activity in male rats and aniline hydroxylase activity in female rats. No hematological changes were observed. Histological examination revealed that the liver and thyroid were the organs most affected by these chlorinated diphenyl ethers. However, morphological changes were mild even at the highest dose. Based on these results, these 3 PCDE congeners are considered to be only moderately toxic to the rat (Chu *et al.*, 1989).

Chu *et al.* (1990), investigated 3 additional PCDE congeners frequently found as environmental contaminants: 2,2′,4,4′,5-pentachlorodiphenyl ether, 2,2′,4,4′,5,5′-hexachlorodiphenyl ether, and 2,2′,3,4,4′,6,6′-heptachlorodiphenyl ether. The ethers were administered in the diets of male and female rats at levels of 0.5, 5.0, 50, or 500 ppm for 4 weeks. At the highest doses, all 3 isomers produced increased liver weights in both sexes. The pentachloro- congener produced an increase in hepatic APDM activity, while the hexachloro-congener caused a significant increase in APDM, aniline hydroxylase, and EROD activities. The heptachloro- congener caused a significant increase in EROD activity and, at the highest dose, a significant reduction in circulating lymphocytes in males. All 3 congeners produced morphological changes in the liver and thyroid. Chu *et al.* (1990), calculated a no-effect level (NOEL) of approximately 5 to 50 ppm orally. They considered that these 3 congeners were only moderately toxic to rats.

In these studies, Chu *et al.* (1989, 1990), also investigated the uptake and

distribution of these PCDE congeners in the rat. The results, which are consistent with those of Newsome *et al.* (1983), showed that level of PCDEs deposited in liver and adipose tissue were dose dependent with adipose tissue having 10 times the PCDE accumulation when compared to liver (Chu *et al.*, 1990).

Howie *et al.* (1990), investigated the immunosuppressive and monooxygenase induction activities of 8 PCDE congeners in C57BL/6N mice. They measured the dose-response suppression of the splenic plaque-forming cell (PFC) response to SRBCs and the induction of hepatic microsomal AHH and EROD activities. Their studies indicate that as *ortho*-chloro substituents are added, activity in PCDEs is not reduced to the same extent as with PCBs. Their studies showed that some coplanar congeners were less immunotoxic than their mono*ortho* analogs, and similar results were observed for their enzyme induction potencies. For the corresponding PCB congeners, the coplanar compounds were significantly more active than their mono*ortho* analogs. In addition, 2 di*ortho*-substituted compounds were also immunotoxic at a dose of 400 μmol/kg whereas, their PCB analogs were inactive. They postulate that the phenyl rings in PCDEs are farther apart than in PCBs due to the intervening ether linkage. Because of this distance, they suggest that the effects of *ortho* substituents are diminished in PCDEs when compared with PCBs (Howie *et al.*, 1990). The reduced Ah receptor agonist activity associated with increased *ortho* substitution in PCBs has been attributed to the reduced coplanarity between the 2 phenyl groups resulting the subsequent loss of binding affinity for the Ah receptor.

Synthesis. Sundström and Hutzinger (1976) and Garå *et al.* (1981), reviewed the

most commonly used methods for synthesizing PCDEs. The most convenient route for synthesizing chlorinated diphenyl ethers utilizes the coupling of a diphenyliodonium salt with a suitable chlorophenol. Of 209 possible congeners, 143 can be synthesized using this method (Garå *et al.*, 1981). Direct halogenation of diphenyl ether is another method for the synthesis of PCDEs, and this reaction is catalyzed by Lewis acids such as $AlCl_3$. This synthetic route is of limited utility because it results in the formation of a complex mixture from which only a limited number of isomers can be isolated (Garå *et al.*, 1981). Additionally, PCDEs can be synthesized via the Ullmann reaction by coupling a phenolic salt with a halogenated benzene. By altering the substitution pattern on the phenol and the benzene, different congeners can be synthesized (Garå *et al.*, 1981). The Ponomarkenko reaction, which is carried out by treating a nitro- or a sulfonyl-substituted diphenyl ether with a high boiling halogenated aliphatic hydrocarbon at high temperature, can also be used to synthesize PCDEs (Garå *et al.*, 1981). Using the Sandmeyer reaction, the nitro group on a substituted diphenyl ether can be reduced to an amino group which in turn can be replaced by a chlorine or hydrogen via diazotization. A PCDE can be nitrated to introduce a nitro group into the ring. The nitro group can then be transformed to the desired PCDE congener using one of the reactions described above (Garå *et al.*, 1981). Howie *et al.* (1990), used direct conversion of a nitro group into a chloro group by treatment with dichlorophenylphosphine, chlorine gas, and phenylphosphonic dichloride. They synthesized various PCDEs with an overall yield > 40%.

Teratogenicity of 2,3,7,8-Tetrachorodibenzo-p-dioxin. Because of the structural similarity between PCDEs and their corresponding PCDDs, it is of interest to review

information currently available on PCDD teratogenicity. Studies have shown that 2,3,7,8-TCDD is an extremely potent inducer of cleft palate and hydronephrosis in mice (Poland and Glover, 1980), although differences in sensitivity of mice to 2,3,7,8-TCDD have been identified. C57BL/6J mice are a sensitive strain whereas ADR/J mice represent a nonsensitive strain (Pratt *et al.*, 1984). Poland and Glover (1980) reported that in 9 out of 10 inbred strains studied, the susceptibility to cleft palate formation produced by 2,3,7,8-TCDD followed the strain distribution of the Ah locus.

When 100 μg/kg of 2,3,7,8-TCDD was administered to C57BL/6J mice (sc) on gestation day 8, 9, or 10, a high percentage of cleft palate was observed. However, 2,3,7,8-TCDD did not significantly induce cleft palate in mice when administered on day 13 (Pratt *et al.*, 1984). In a similar study, the incidence of fetal hydronephrosis and cleft palate in C57BL/6J mice treated by oral gavage with 62 nmol/kg 2,3,7,8-TCDD on days 9 and 10 of gestation was 61.8% and 87.8% respectively (Biegel *et al.*, 1989). Haake *et al.* (1987), dosed C57BL/6J mice with 20 μg/kg 2,3,7,8-TCDD (oral gavage) on day 10 of gestation. At this dose, 61.1% of the fetuses had cleft palate and 87.8% had hydronephrosis. There were no apparent signs of maternal toxicity at this dose.

Gestation days 11 and 12 are the most sensitive times for induction of cleft palate by 2,3,7,8-TCDD in C57BL/6N mice (Birnbaum *et al.*, 1985), and a steep dose-response curve was observed for this effect after administration of a single dose on day 11. A 12 μg/kg dose administered to dams caused cleft palate in 36% of the fetuses. No affected fetuses were detected at 0 or 10 μg/kg, while a dose of 14 μg/kg resulted in more than 70% affected fetuses.

Renal lesions induced by 2,3,7,8-TCDD are most clearly described as hydronephrosis and are characterized by a grossly dilated renal pelvis. Moderate to severe renal damage was evident when 10 μg/kg of 2,3,7,8-TCDD was administered orally to C57BL/6N mice on days 10 through 13 (Birnbaum *et al.*, 1985).

Mouse embryos incubated in buffered Delbucco's Modified Eagles/Ham's F12 medium for 24 hr with 0.0 to 1.0 μg/ml 2,3,7,8-TCDD were evaluated for cranial facial anomalies, heart rate, and crown-rump length. There were no significant differences between embryos treated with 0.001 to 0.005 μg/ml 2,3,7,8-TCDD and control embryos. With concentrations of 0.05 and 0.01 μg/ml, defects in the head and mandibular arch were observed. Concentrations of 1.0 and 0.1 μg/ml 2,3,7,8-TCDD in the culture media were lethal to embryos within min (Nuzzo *et al.*, 1989).

It has been suggested that the toxic effects produced by 2,3,7,8-TCDD are mediated by its binding to the cytosolic Ah receptor protein. Poland and Glover (1980) examined the effects of 2,3,7,8-TCDD on 3 inbred strains of mice: C57BL/6J mice, which have a high affinity Ah receptor and are sensitive to TCDD-mediated-induction of AHH activity; DBA/2J mice, which have a lower affinity receptor and are less sensitive to the induction of AHH activity by TCDD; and hybrid B6D2F_1/J mice. Their studies showed that 2,3,7,8-TCDD produced a dose-related incidence of cleft palate formation in fetuses of C57BL/6J mice, and that the overall response in the 3 strains corresponded to binding affinity for the Ah receptor. A single dose of 30 μg/kg of 2,3,7,8-TCDD on day 10 of pregnancy produced a 54% incidence of cleft palate in C57BL/6J fetuses. The incidence of cleft palate was 13% with B6D2F_1/J fetuses, and 2% in DBA/2J fetuses.

The progeny of a B6D2F$_1$/J X DBA/2J mating were phenotyped (as Aa or aa), and the mice heterozygous for the high affinity receptor were more sensitive to thymic atrophy. The investigators also demonstrated that five inbred strains of mice with a low affinity receptor developed only 0-3% incidence of cleft palates when dosed with 30 μg/kg 2,3,7,8-TCDD. On the other hand, 4 out of 5 inbred strains with a high affinity receptor developed a 50% or greater incidence of cleft palate (Poland and Glover, 1980).

2,3,7,8-TCDD appears to induce cleft palate through a direct, receptor-dependent effect on the embryonic palatal shelves (Abbott and Birnbaum, 1990). There is a differential sensitivity of various mouse strains to 2,3,7,8-TCDD-induced cleft palate which correlates well with the level of 2,3,7,8-TCDD receptors in the cytosol of liver and thymus (Poland and Glover, 1980).

2,3,7,8-TCDD is an unusual inducer of cleft palate in mice. Most chemicals and drugs induce cleft palate in the mouse when administered between days 11 and 14 of gestation. 2,3,7,8-TCDD, however, produces a high percentage (>95%) of cleft palate when administered as a single sc dose (100 μg/kg) on any day from days 8 through 10 of gestation (Pratt *et al.*, 1984). Studies with rats have provided somewhat different results. Sparschu *et al.* (1971), administered 2,3,7,8-TCDD orally to pregnant rats on days 6 through 15 of gestation at levels of 0 (control), 0.03, 0.125. 0.5, 2.0, and 8.0 μg/kg/day. No adverse effects on fetuses were noted at the 0.03 μg/kg dose level. There was, however, a dose-related increase in fetal intestinal hemorrhage and fetal mortality at the 0.125 to 2.0 μg/kg dose levels. Severe maternal toxicity and embryo toxicity were evident at the 8.0 μg/kg/day dose with maternal weight loss and early resorptions

occurring in all cases.

In another study, pregnant Holtzman rats received 2,3,7,8-TCDD in corn oil (0, 1.5, 3, 6, or 18 μg/kg, po) on gestation day 10, and pregnant Golden Syrian hamsters received 2,3,7,8-TCDD in corn oil (0, 1.5, 3, 6, or 18 μg/kg, po) on gestation day 9. At 18 μg/kg, the fetal mortality rate was 72% for rats and 60% for hamsters. In neither case was any overt maternal toxicity detected. Cleft palate and hydronephrosis were observed in a few viable rat fetuses, but gastrointestinal hemorrhage was the most sensitive indicator of *in utero* exposure to 2,3,7,8-TCDD, with an incidence of 41% at the 1.5 μg/kg dose. In hamsters, kidney abnormalities were the most sensitive indicator of 2,3,7,8-TCDD exposure, with an incidence of 42% at a dose of 3.0 μg/kg (Olson and McGarrigle, 1989).

In post-implantation whole embryo culture, no abnormal development was seen in rat embryos with concentrations up to 90 *n*g/ml, although it was shown that 2,3,7,8-TCDD did, in fact, reach the developing embryo. After *in vitro* incubation of the embryos for 48 hr with 39 ng/ml 2,3,7,8-TCDD in the medium, the content of the dioxin was: amniotic fluid 6 ng/ml; yolk sac, 44 ng/g ww; and embryo, 10 ng/g ww (Neubert *et al.*, 1987).

Induction of cleft palate was not reported in teratology studies where 2,3,7,8-TCDD (≤ 8.0 μg/kg/day) was administered orally to pregnant rats on gestation days 6 through 15 (Sparschu *et al.*, 1971). The reason for the relative insensitivity of the rat to induction of cleft palate as compared to the mouse is unknown. Studies show that the mouse may be a more sensitive model for evaluating specific toxic developmental

responses following exposure to structurally related polyhalogenated aromatic hydrocarbons such as the dioxins, furans, biphenyls, and diphenyl ethers (Couture *et al.*, 1989). In more recent studies (Abbott and Birnbaum, 1990; Couture *et al.*, 1990), F344 rats were exposed to 2,3,7,8-TCDD in organ culture. This eliminated the possible effects of maternal toxicity on induction of cleft palate. An altered differentiation of rat medial epithelium, similar to that reported for 2,3,7,8-TCDD-exposed C57BL/6N mouse medial cells *in vivo* and *in vitro*, was reported. However, much higher concentrations of 2,3,7,8-TCDD were required to obtain these responses in the rat. Rat palatal shelves were 200 times less sensitive to 2,3,7,8-TCDD than were mouse palatal shelves. The same effect is induced in medial epithelium of rats and mice, but cleft palate is not seen in rats because the level required to produce the toxic effects would result in maternal and embryo toxicity, including fetal death (Abbott and Birnbaum, 1990).

The response of human palatal shelves examined in an *in vitro* organ culture system was similar to that of the rat in terms of cleft palate induction. On a cellular level the altered differentiation of medial cells was identical in rat, mouse, and human organ culture systems. The sensitivity of human shelves was approximately 200 times less than that of the mouse and most closely approximated that of the rat (Couture *et al.*, 1990). The response of a particular organ or cell to the effects of 2,3,7,8-TCDD requires the presence of a functional Ah receptor. Although this functional receptor is necessary, it is not sufficient for the induction of a biochemical response by 2,3,7,8-TCDD and related compounds (Safe, 1990).

Teratogenicity of PCBs. Like the PCDEs, the closely related PCBs contain from

1 to 10 chlorine substituents and comprise a family of 209 congeners and isomers. The effects of PCB mixtures on reproduction have been investigated in several species. These chemicals are known to cross the placenta in some mammals, including humans (Kimbrough *et al.*, 1978). According to Safe (1989), there are quite likely some barriers to transplacental exposure of these toxins since PCB levels in maternal blood were shown to be considerably higher than levels in umbilicus blood and amniotic fluid. Nevertheless, the passage of PCBs across the placenta, coupled with a large amount of data that PCBs can be embryotoxic, is reason for concern (Marks *et al.*, 1981).

Commercial PCB mixtures are known to be very heterogenous. This fact makes toxicological assessment difficult. Differences in the physical and chemical properties of these congeners influence their absorption, distribution, metabolism, and excretion rates. For this reason, it is important to study specific PCB congeners and to determine which are least likely to cause adverse effects (Marks *et al.*, 1981).

The possible teratogenic effects of PCBs in mice have been investigated by Marks *et al.* (1981). Pregnant outbred albino (CD-1) mice were given 3,3′,4,4′,5,5′-hexachlorobiphenyl by gavage on days 6 through 15 of gestation. Doses ranged from 0.1 to 16 mg/kg/day. On day 18, mice were sacrificed and fetuses examined for external, visceral, and skeletal malformations. A significant increase in fetal deaths occurred with doses of 4, 8, and 16 mg/kg/day, and a significant decrease in average fetal weight and an increase in the percentage resorptions occurred with 8 and 16 mg/kg/day doses. With doses of 2, 4, 8, and 16 mg/kg/day, the average percent of malformed fetuses per litter increased by 11.7%, 36.9%, 65.5%, and 60.6%, respectively. In the vehicle control group,

0.9% of fetuses were malformed. The predominant malformations detected were cleft palate and hydronephrosis. These defects increased significantly at doses as low as 2 and 4 mg/kg/day. Other signs of embryotoxicity such as liver discoloration and small renal papillae, were seen at the 1 mg/kg/day dose, although no significant malformations occurred at this dose level (Marks *et al.*, 1981).

Marks *et al.* (1981), tested the developmental toxicity of several symmetrical PCB congeners, and their results showed that only the coplanar congeners substituted in the 3, 3′, 4, and 4′ positions were active. Subsequent studies by Marks *et al.* (1989), supported their earlier conclusions. They investigated the teratogenicity of 4,4′-dichlorobiphenyl, 3,3′,4,4′-tetrachlorobiphenyl, 3,3′,5,5′-tetrachlorobiphenyl, and a non-chlorinated biphenyl, 3,3′,4,4′-tetramethylbiphenyl. These compounds were administered to outbred albino (CD-1) mice by gavage on days 6 through 15 of gestation. The mice were sacrificed on day 18 and fetuses examined for external, visceral, and skeletal malformations. At 64 mg/kg/day, 4,4′-dichlorobiphenyl showed no developmental toxicity, and 3,3′5,5′-tetrachlorobiphenyl and 3,3′4,4′-tetramethylbiphenyl showed neither maternal nor developmental toxicity. Administration of 3,3′4,4′-tetrachlorobiphenyl, however, was followed by a significant increase in the average percentage of malformed fetuses per litter with 4, 8, 16, 32, and 64 mg/kg/day resulting in 7.2%, 9.8%, 25.4%, 50.0%, and 75.0% malformed fetuses, respectively. In the vehicle control group, 1.1% of fetuses were malformed. Cleft palate and hydronephrosis, which increased significantly at doses of 4 mg/kg/day and above, were the predominant malformations which were detected. None of the doses of 3,3′,4,4′-tetrachlorobiphenyl were lethal to the dams (Marks *et al.*,

1989).

Oral gavage of pregnant C57BL/6 mice with 3,3′,4,4′-tetrachlorobiphenyl on day 11, 12, or 13 of gestation, with doses ranging from 25 to 100 mg/kg, resulted in embryolethal effects, cleft palate, dilated kidney pelvis, and hypoplasia of the thymus (d'Argy *et al.*, 1987). Oral administration of 3,3′4,4′-tetrachlorobiphenyl to Sprague-Dawley rats with 3 or 10 mg/kg/day on gestation days 6 through 18 was embryolethal and caused accumulation of blood in the amniotic fluid, intestines, and stomachs of the fetuses (Wardell *et al.*, 1982).

Studies to determine the possible effects of selected PCBs as 2,3,7,8-TCDD antagonists in C57BL/6J mice have recently been reported. At oral doses as high as 750 to 1000 μmol/kg, on days 9 and 10 of gestation, 2,2′,4,4′,5,5′-hexachlorobiphenyl did not cause fetal cleft palate. However, at 750 μmol/kg it did cause a significant increase (52.9%) in fetal hydronephrosis (Beigel *et al.*, 1989). Co-treatment of these mice with 3.7 nmol/kg 2,3,7,8-TCDD and 2,2′4,4′5,5′-hexachlorobiphenyl showed that the PCB congener partially antagonized 2,3,7,8-TCDD-mediated cleft palate. Using 2,3,7,8-^{3}HTCDD, it was shown that some of the effects of the hexachlorobiphenyl on 2,3,7,8-TCDD-mediated cleft palate may be due to decreased levels of 2,3,7,8-TCDD in the fetal palates after co-treatment with the two substances (Biegel *et al.*, 1989).

The effects of commercial PCB mixtures on colonies of *Hydra oligactis* have been investigated by Adams and Haileselassie (1984). Aroclor® 1016 was more acutely toxic to hydra than the more widely used and higher chlorinated Aroclor® 1254. Aroclor® 1016 also inhibited the regeneration of tentacles by hydra and the relative potency ratio

of Aroclor® 1016/Aroclor® 1254 was > 4. These differences in potency could be related to the relatively low solubility and bioavailability of the more highly chlorinated PCBs which predominate in Aroclor® 1254. These results are consistent with those reported by Ewald *et al.*, (1976) who demonstrated that the toxicity of PCBs to *Euglena gracilis* is inversely proportional to the percent chlorination. Aroclor® 1016 is also the more water soluble of the 2 PCB mixtures and should have a wider distribution among the aqueous compartments of cells and tissues (Adams and Haileselassie, 1984).

In Vitro Assays for Teratogenesis. A number of short-term developmental toxicology *in vitro* assays have been designed in response to the recognition that *in vivo* teratology testing procedures cannot adequately meet teratology testing demands (Sabourin *et al.*, 1985). The term "developmental toxicity" covers any detrimental effect during embryonic stages of development caused by any toxic exposure. Those substances which cause irreversible structural or functional abnormalities in offspring, but not death, are known as teratogens. These teratogenic agents cause adverse effects to the conceptus at exposure levels that are not toxic to the mother (Manson, 1986).

In studying developmental toxicology, several basic concepts must be understood. First, a potential teratogen may exhibit a degree of specificity in producing a particular malformation. Even though the mechanism of action may be unknown, there is a correlation between the malformation produced and specific metabolic events in the development of the affected organs. Second, dosage is of utmost importance. Not all doses of a teratogen are teratogenic. There can be a low level that permits normal development and a high level which is embryolethal and even maternally toxic or

maternally lethal. Between these points there can exist a teratogenic range. Embryonic age is another factor of extreme importance. Susceptibility to teratogenic insult varies greatly during the course of gestation. The time that insult occurs can determine which embryonic tissues will be affected. In the rat, the onset of teratogenic susceptibility occurs at approximately day 8 (Wilson, 1965a).

Minimal doses of a test compound should be used to identify the most susceptible time for a given malformation to occur, since higher doses may introduce other defects and give ambiguous results. Some organs, such as the palate, may show more than one susceptible period. Such susceptibility generally indicates that more than one underlying embryological process is involved (Wilson, 1965b).

Developing a series of tests to be used in a protocol to prescreen potentially toxic agents could help solve the problem of how to test hundreds of thousands of chemicals for developmental toxicity. Most substances are potentially capable of disrupting development of the embryo, but in many cases this disruption occurs only at doses high enough to be overtly toxic to the mother. These substances, which are termed coaffective teratogens, generally affect both the mother and the developing embryo at approximately the same exposure levels (Johnson and Christian, 1985).

The difficulties inherent in evaluating the teratogenic potential of all chemicals and combinations thereof to which women of child-bearing age may be exposed are well recognized. The overwhelming majority of chemicals already in commerce or being brought into use each year have not been evaluated for their potential to adversely affect *in utero* development (Johnson *et al.*, 1987). There are neither enough trained people nor

adequate facilities to care for the number of pregnant rodents required to conduct *in vivo* studies on the hundreds of new chemicals that are developed each year, not to mention those chemicals already present in the commercial inventory (Johnson and Christian, 1985). Increased testing requirements are further complicated by factors such as the already high and ever increasing cost of animal care and testing, the large amount of time required for animal testing, and a rising concern over the rights of animals used for research (Goss and Sabourin, 1985). Two *in vitro* systems that may well be used as part of a prescreen for potential teratogenic chemicals are the hydra assay and post-implantation whole embryo culture.

CHAPTER III

EXPERIMENTAL ASSESSMENT OF DEVELOPMENTAL TOXICITY OF CHLORINATED DIPHENYL ETHERS IN HYDRA

The hydra assay has been developed as a simple and efficient means for prescreening substances for developmental toxicity. This assay can provide a quick, inexpensive method to identify those few substances worthy of more intense evaluation in the developing fetus (Johnson and Gabel, 1983b). It circumvents many problems associated with whole animal studies, which are costly, labor-intensive, and generally quite lengthy. Because the artificial embryos develop in a manner highly analogous to mammalian systems, it is possible that they undergo many of the same teratogenic mechanisms which affect mammalian systems, but do so over a shorter period of time.

Hydra are the lowest form of animal life composed of complex organs and tissues. At the same time, they are the highest form of animal life capable of total whole-body regeneration (Johnson *et al.*, 1982). *Hydra attenuata* is relatively simple to feed and maintain. It most commonly undergoes vegetative reproduction by budding. This small fresh-water coelenterate can be dissociated into its component cells which can then be reaggregated into small pellets. If these pellets are placed into a proper supportive medium, they will develop into new, normal adult hydra within 2 weeks (Johnson and Gabel, 1983a). In so doing, the cells pass through the stages expected of any developing system: proliferation of cells; induction and response; creation of organ fields; spatial orientation; membrane changes; directional migration; metabolic changes; cellular

morphogenesis; histogenesis; and organogenesis (Collins, 1987).

These embryos can be exposed to test agents at various concentrations to determine the minimum dose which is capable of interfering with development. The developmental stage at which the perturbation occurs is not important. What is important is the exposure level which produces any adverse effect on the developing embryo. Similarly, the adult hydra can be exposed to various concentrations of test agent. From the minimum affective concentrations for the adult and the embryo, the adult/developmental (A/D) ratio can be determined (Johnson and Christian, 1985). While the A/D ratio of hydra is not predictive of the A/D ratio in mammals in every instance, it is more than adequately predictive for separating coaffective from intrinsic developmental toxins (Johnson and Christian, 1985).

Chemicals in which the A/D ratio ranges from 1 to 3 are referred to as coaffective teratogens and demonstrate near equal toxicity to both the maternal organism and the embryo. Those chemicals with an A/D ratio greater than 10 are considered non-coaffective or potent teratogens and should be targeted for additional studies using classical teratology experiments. Chemicals with A/D ratios intermediate to the coaffective and non-coaffective ranges are considered moderate teratogens. These substances could be targeted for less intensive teratology experiments to better define their developmental toxicity (Johnson, 1987).

With the hydra assay, it is possible to examine a large number of test agents at multiple exposure concentrations. Adults and embryos are exposed to a series of log and then 1/10 log concentrations of test compounds ranging from 10^{4} to 10^{-3} µg/ml. Those

that are determined to be coaffective agents would not warrant further elaborate developmental toxicity testing. Attention could then be focused on testing those substances found to be uniquely hazardous to the developing embryo.

Materials and Methods

Chemicals. PCDEs and PCBs were provided by Dr. Stephen Safe, Department of Veterinary Pharmacology and Physiology (Texas A&M University). Amikacin was obtained from Bristol Laboratories, Syracuse, NY. Dimethylsulfoxide (DMSO) and all other chemicals were from Sigma (St. Louis, MO.). Brine shrimp eggs were purchased from Carolina Biological Supply, Burlington, NC. The original stock of *Hydra attenuata* was a gift from Dr. E. Marshall Johnson.

Hydra Assay. Hydra were maintained in a medium of 1.0 mM $CaCl_2$ dihydrate, 0.458 mM TES buffer, and 0.012 mM EDTA dissolved in deionized-distilled water. The pH was adjusted to 6.9 ± 0.1. Ambient temperature was maintained at 18°C. Hydra were fed freshly hatched *Artemia nauplii* (brine shrimp) once a day. The shrimp eggs (approximately 15 ml) were hydrated in 1% NaCl for approximately 2 days at room temperature. Eggs were aerated throughout the hatching period. Prior to feeding to the hydra, brine shrimp were siphoned into a fish net (125 to 140 mesh) and disinfected for 10 min in a tetraglycine hydroperiodide solution (2 tablets/400 ml 1% NaCl) then rinsed twice with deionized-distilled water to remove any traces of salt or tetraglycine hydroperiodide. Hydra were placed in fresh medium no sooner than 30 min after each feeding to remove digested materials, exoskeletons, and unconsumed brine shrimp and eggs from their environment. Hydra were treated once a week with tetraglycine

hydroperiodide (1 tablet in 3 to 3.5 liters of hydra medium).

All adult hydra used for the experiment were treated with tetraglycine hydroperiodide (described above) 60 to 72 hr prior to beginning the assay. After a 24 hr starvation period, groups of 3 adult hydra were placed into glass petri dishes containing 4 ml of hydra medium plus Amikacin sulfate (60 µl/100ml) with the pH adjusted to 6.9 ± 0.1. Various concentrations of PCDEs or PCBs dissolved in DMSO were added to the medium. The solvent concentration in the medium in the petri dishes was maintained uniformly at 1%. Control and solvent control assays were run simultaneously with each test compound (Table 1).

Hydra were examined for signs of a toxic response at 4, 20, 28, 44, 68, and 92 hr. After each observation, hydra were placed in fresh medium containing the appropriate test compound. Toxicity was assessed during each observation by examining for the presence of clubbed tentacles, shortened tentacles, and tulip formation (Fig. 4). Tulip formation was considered to be the toxic endpoint for the adult assay (Johnson and Gabel, 1983a).

Results

Eight PCDEs and PCBs were tested at the highest concentrations possible with solubility in hydra medium the limiting factor. In no case was the toxic endpoint or "tulip" stage reached. Adult hydra exposed to 2,3′,4,4′,5-pentachlorobiphenyl (100 µg/ml) displayed clubbed tentacles at 68 hr, and shortened tentacles appeared at 92 hr. Hydra exposed to 2,3′,4,4′,5-pentachlorodiphenyl ether (100 µg/ml) showed clubbing at 68 hr which persisted throughout the experiment. Adult hydra were unchanged after exposure to 3,3′,4,4′,5-pentachlorobiphenyl (100 µ/ml) (Fig. 5).

TABLE 1

Test substances evaluated using the hydra assay.

Group	Test Substance	Concentration
1	Control	
2	Solvent Control[a]	
3	3,3′,4,4′-tetra-CDE	10 µg/ml
4	2,3′,4,4′,5-penta-CDE	10 µg/ml
5	2,3′,4,4′,5-penta-CDE	100 µg/ml
6	2,3′,4,4′,5-penta-CB	10 µg/ml
7	2,3′,4,4′,5-penta-CB	100 µg/ml
8	3,3′,4,4′,5-penta-CDE	10 µg/ml
9	3,3′,4,4′,5-penta-CB	10 µg/ml
10	2,3,3′,4,4′,5-hexa-CDE	10 µg/ml
11	2,2′,4,4′,5,5′-hexa-CDE	10 µg/ml
12	2,2′,3,3′,4,4′,5,6,6′-nona-CDE	1.0 µg/ml
13	2,2′,3,3′,4,4′,5,6,6′-nona-CDE	10 µg/ml

[a] 1% DMSO

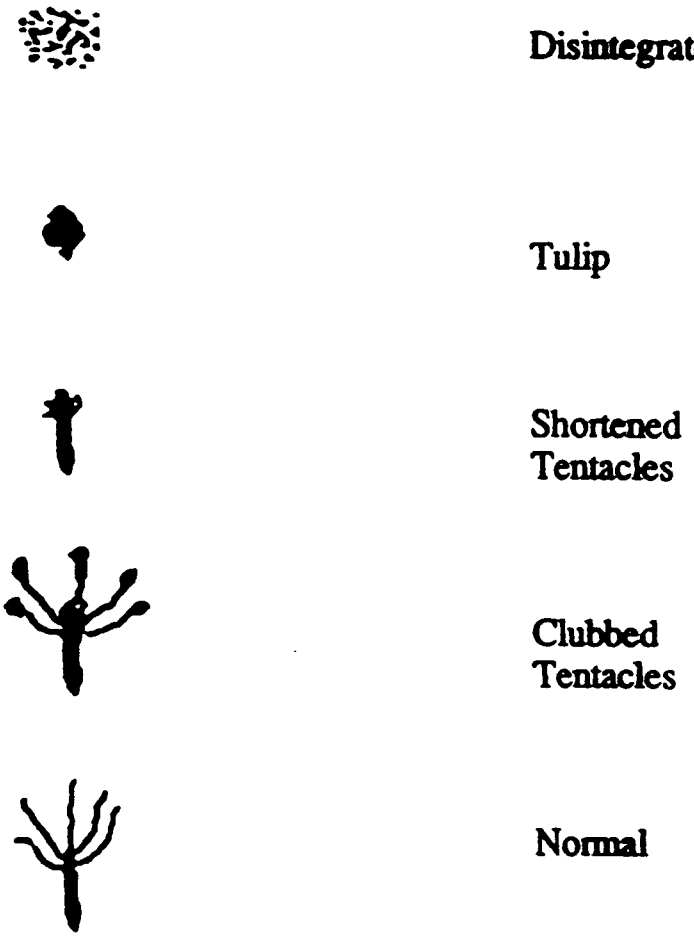


FIG. 4. Response of adult hydra to a toxic substance.

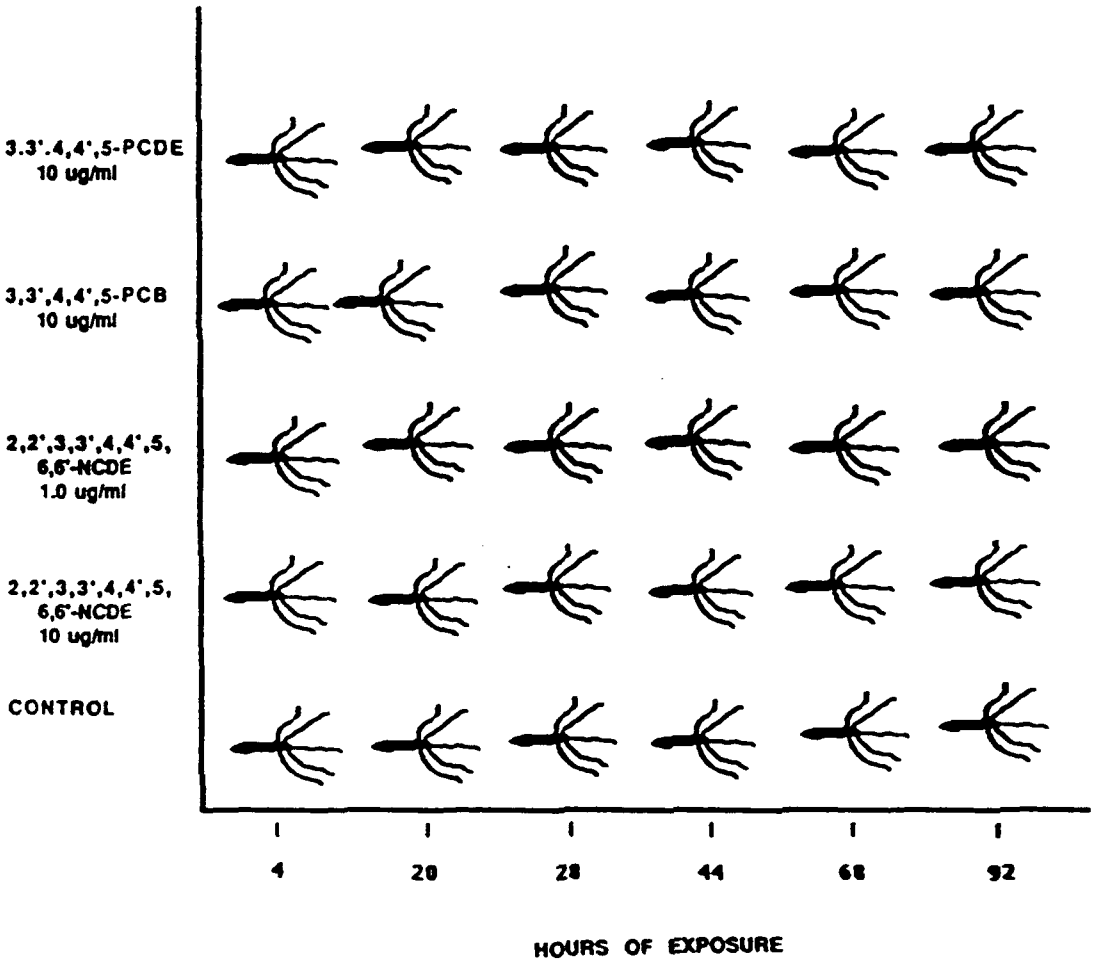


FIG. 5. Response of *Hydra attenuata* to selected polychlorinated diphenyl ethers and polychlorinated biphenyls.

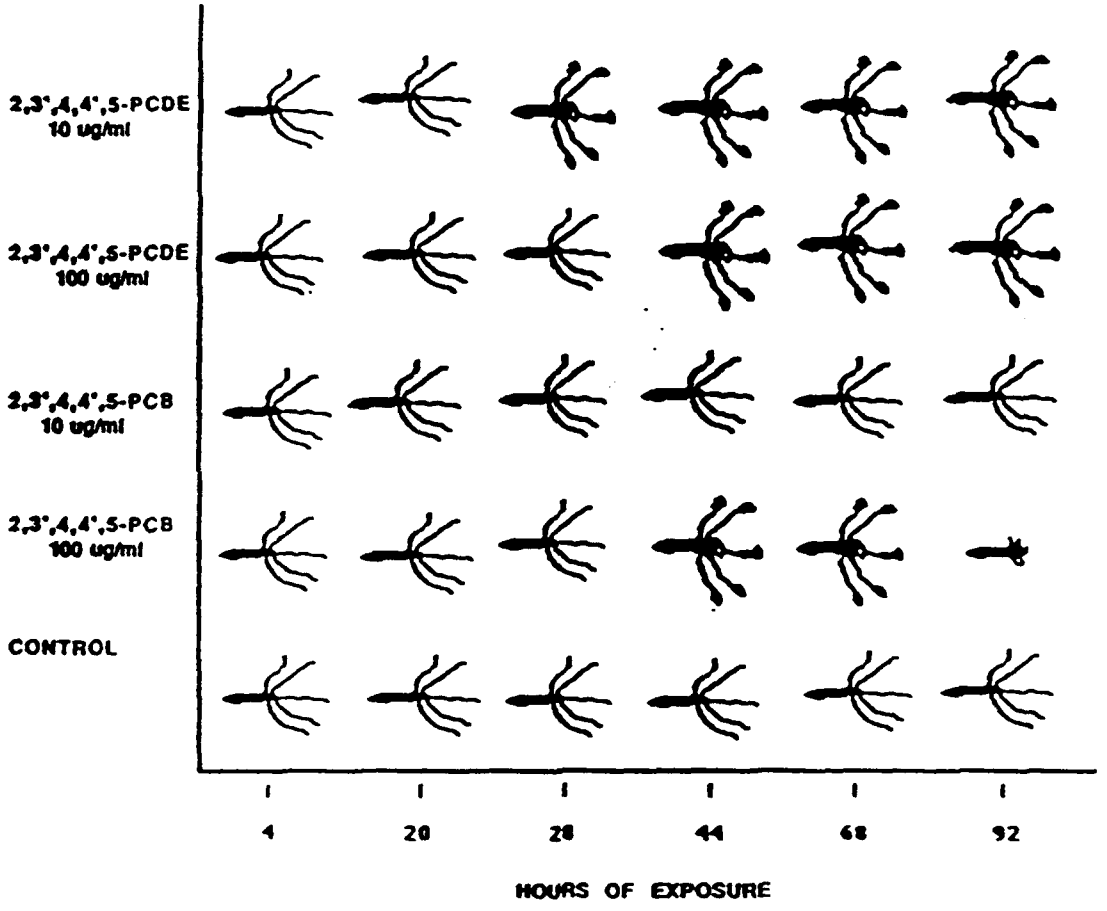


FIG. 5. Continued

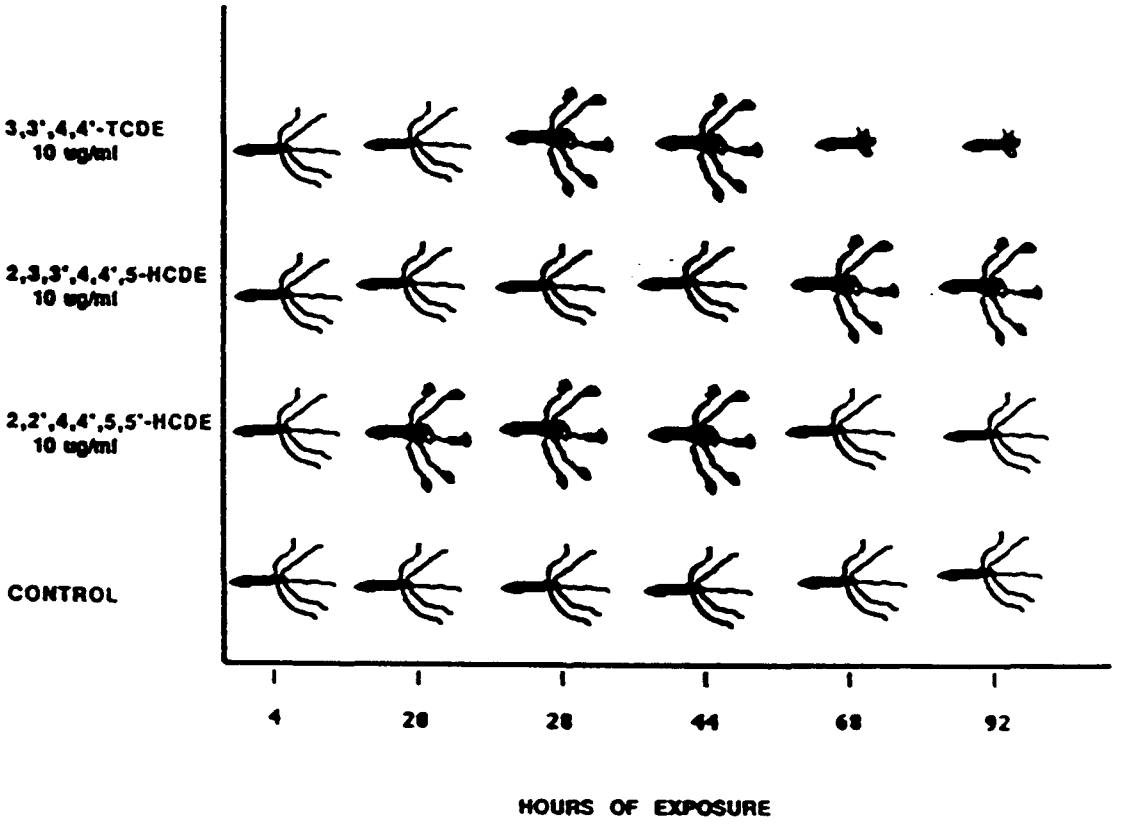


FIG. 5. Continued

Hydra exposed to 10 µg/ml 2,3′,4,4′,5-pentachlorodiphenyl ether showed persistent clubbed tentacles, after a period of 28 hr. With 2,3,3′,4,4′,5-hexachlorodiphenyl ether (10 µg/ml), clubbed tentacles were observed at 68 hr, and remained the same throughout the assay period. With 10 µg/ml 3,3′,4,4′-tetrachlorodiphenyl ether, clubbed tentacles were observed at 28 hr and shortened tentacles were observed at 68 hr. Interestingly, with 10 µg/ml of 2,2′,4,4′,5,5′-hexachlorodiphenyl ether, hydra exhibited clubbed tentacles at 20 hr, but by 68 hr, they had recovered and normal development continued for the remainder of the 92 hr period (Fig. 5).

These chemicals were not tested on embryos because the toxic endpoint was not reached with adult assays. Consequently, A/D ratios were not determined.

Discussion

Although they did not elicit a toxic endpoint, adult hydra responded to the test chemicals by forming clubbed or shortened tentacles. These responses cannot be considered as a toxic endpoint because recovery from these stages is possible. Once the hydra have reached the tulip stage, recovery does not occur. It is possible that the toxic endpoint would have been reached if solubility problems had been overcome and higher concentrations of the test compounds had been used. Studies to evaluate different solvent systems for delivering compounds that are only sparingly soluble in water have been conducted with the hydra assay. Solvents found thus far to be compatible with the assay are 1% (v/v) ethanol, 0.5% (v/v) DMSO, and 0.1% (w/v) carboxymethyl cellulose. Moreover, solvent systems of DMSO:propylene glycol (3:7) and ethanol:glycerol:hydra medium (2:3:5) have been useful for testing agents not adequately soluble in hydra

medium alone (Johnson *et al.*, 1988). Studies to evaluate the potential toxic effects of these and other polychlorinated aromatic compounds in the hydra assay using some of the alternative solvent systems would be worthy of investigation.

Because the metabolites rather than the parent chemical may represent the greater hazard potential, a developmental toxicity prescreening system is more useful if it has the capability to produce metabolites similar to those formed in standard pregnant laboratory animals. Kaji *et al.* (1983), demonstrated that hydra showed no endogenous P-450 activity as measured by double-beam spectrophotometry, nor was any detectable activity elicited by exposure to several known mixed function oxidase inducing agents. In later studies, Newman *et al.* (1990), tested the proteratogen, cyclophosphamide, in the hydra assay in the presence and absence of an *in vitro* metabolic activating system consisting of rat hepatic microsomes (0.06 *n*mol P-450/ml), 500 μM NADPH, and 25 μM MgCl. Inclusion of this bioactivating system increased the toxicity of cyclophosphamide to both adults and developing embryos by a factor of more than 200. The addition of metabolic activation capability to an *in vitro* assay, while not essential, markedly enhances its utility in developmental toxicity safety evaluations (Newman *et al.*, 1990).

The hydra assay is a predictive and reliable test for evaluating diverse types of chemicals (Johnson *et al.*, 1987). The assay has a distinct advantage over some other *in vitro* protocols in assessing teratogenicity in that it has a clearly defined and reliable endpoint (Sabourin *et al.*, 1985).

A large and diverse group of chemicals has been evaluated in the hydra assay, and results are consistent with those of standard developmental toxicity evaluations made in

groups of pregnant mammals. Hydra correctly identified each of the substances previously found by *in vivo* tests to be uniquely hazardous to *in utero* development. Its overall accuracy has been > 90%, and all errors have been false positives. When higher level tests indicated that a chemical was uniquely hazardous to the conceptus, in no case did the hydra assay indicate that it was not. The hydra assay provided A/D ratios in agreement with ratios for 57 of 61 chemicals derived from Segment II studies (Johnson *et al.*, 1988). When the hydra assay was not predictive, it tended to overestimate the developmental toxicity hazard potential. Moreover, when the same chemical was tested in hydra independently by a second laboratory, the A/D ratio varied only slightly indicating a high degree of interlaboratory consistency of the results (Johnson *et al.*, 1988).

In our study, had we been able to test higher concentrations of the test substances, the toxic endpoint may have been reached. Had that been the case, we would have continued the assay using artificial embryos. Without comparative data with hydra embryo studies, it is not possible to determine A/D ratios. The hydra assay may represent a unique prescreen for prioritizing chemicals for further teratological study.

CHAPTER IV

EXPERIMENTAL ASSESSMENT OF THE DEVELOPMENTAL TOXICITY OF CHLORINATED DIPHENYL ETHERS IN THE RAT

Post-implantation whole embryo culture is a teratological prescreening bioassay which combines the use of mammalian embryos in an environment similar to that observed *in vivo*, with the ease, reproducibility, and cost-effectiveness of cell and organ culture methodologies. The utility of this *in vitro* culture system is based on the fact that teratogen-induced abnormalities observed *in vivo* can also be noted in embryos in an embryo culture system and in embryos cultured with serum from adult animals treated with the teratogens (Sadler *et al.*, 1982).

In the development of the mammalian embryo, all the main organ systems appear soon after the embryo is implanted in the uterine wall. For close and continuous observation in many kinds of experimental investigations, it is essential to explant the embryos and grow them in culture. Early attempts to devise culture methods for post-implantation embryos met with many difficulties. However, in the 1960s, there was renewed interest in the possibilities of embryo culture (New, 1978), and this was partly influenced by the increasing concern regarding teratogenesis following the thalidomide disaster. Since then improved methods have been devised and used in a wide range of studies on the physiology and development of the post-implantation embryo. The advantages provided by the whole embryo culture system are associated with the control over the environment of the embryo *in vitro*, and the capability to modulate this

environment with great precision (New, 1990)

Embryo culture is a useful method for eliminating many of the confounding maternal influences such as stress, nutritional effects, and uterine position from the fetal environment (Fantel, 1982). For indirect acting toxins, a metabolic activation can be added to the whole embryo culture to mimic the metabolic events which take place *in utero*. Warner *et al.* (1983), compared *in vivo* and *in vitro* embryonic responses to a known teratogen, hydroxyurea, while controlling drug concentration and exposure time. Control embryos grown *in vivo* and *in vitro* were indistinguishable. The whole embryo culture system produced malformations which mimicked those observed *in vivo*. Cirurel and Schmid (1988) also compared the *in vivo* and *in vitro* responses of 25 compounds. Sixteen known *in vivo* teratogens induced specific malformations in embryos grown in culture. Of the 9 compounds which were negative in *in vivo* rat teratogenicity studies, none caused abnormal morphology in cultured embryos. Their results demonstrated a high predictability of the whole embryo culture system for the compounds tested relative to *in vivo* studies.

Kitchin and Ebron (1984) investigated various solvents for the delivery of water-insoluble compounds in the embryo culture system. At concentrations of 0.1% and 0.5% DMSO, no increased incidence of embryonic abnormalities were noted. However, at 2.5% DMSO, 100% of the embryos were abnormal with a 90% mortality rate. When distilled water was used as a solvent control, 12.5% of the embryos were abnormal, but there was no embryo mortality.

A question of considerable interest with respect to the applicability of the rat

whole embryo culture is whether or not the rat embryo has an enzyme system capable of metabolizing xenobiotics. According to Kitchin *et al.* (1981), the rat embryo has little or no ability to biotransform inactive compounds to more reactive metabolites through mechanisms such as the microsomal MFO enzyme system. Control studies in which co-factors for the monooxygenase enzymes were omitted suggested, but did not prove, that the rodent embryo does not provide sufficient bioactivation of test chemicals to alter growth or morphological development (Shepard *et al.*, 1983). Studies by Juchau *et al.* (1985), on the other hand, suggest that *in utero* induction of P-450-dependent embryonic monooxygenases resulted in the production of embryotoxic metabolites.

A number of studies (Kitchin *et al.*, 1981; Faustman-Watts *et al.*, 1983; Kitchin and Ebron, 1983) have incorporated a source of biotransformed enzymes plus co-factors for particular reactions, directly into the culture medium. The enzyme source most often used is a microsome-rich hepatic fraction. The co-factor, NADPH, serves as an electron donor for phase I reactions (Fantel, 1982).

Whether there is an endogenous embryonic drug metabolizing enzyme system present or not, it is possible to add exogenous adult rat liver microsomes plus co-factors to the assay, to mimic the *in utero* environment. The addition of a metabolic activating system allows for increased sensitivity and versatility in whole embryo culture techniques, and can also be useful in determining the role of maternal metabolism in embryotoxicity and teratogenesis (Kitchin *et al.*, 1981).

Since the *in vitro* whole embryo culture system lacks a pathway for maternal excretion and hence an ability to progressively lower serum concentrations of test

chemicals, the assay could produce a higher number of false positive results. Kitchin and Ebron (1983) investigated saccharin and cyclohexylamine, two agents which are rapidly excreted by the kidneys and which have demonstrated a lack of teratogenicity and embryotoxicity in *in vivo* studies. The results of their *in vitro* tests using whole embryo culture with a microsomal activating system were consistent with published *in vivo* findings. They concluded that the lack of an excretion pathway did not significantly alter the toxicity of these 2 substances (Kitchin and Ebron, 1983).

Definitive parameters for quantitative estimation of embryonic growth and development help realize the maximum potential of whole embryo culture techniques. Brown and Fabro (1981) devised an objective scoring system which provides a precise measure of morphological development. They also examined several parameters as estimates of embryonic growth distinct from development. Yolk sac diameter, crown-rump length, and head length were found to vary with embryonic age as quadratic functions over time. Total embryonic protein, which increased logarithmically with embryonic age, was considered to be the most suitable measure of embryonic growth (Brown and Fabro, 1981).

Materials and Methods

Animals. Mature male and nulliparous female Sprague-Dawley rats (200-225g) were obtained from Harlan Sprague-Dawley Inc., Indianapolis, IN. They were housed at the Texas A&M University Laboratory Animal Resources and Research Facility in a temperature controlled, artificially illuminated room (12 hr light and 12 hr dark) free from any known source of chemical contamination. Food and water were available to animals

ad libitum. After an acclimation period of 7 days, an experienced male was housed with a single female in a filter top, polycarbonate cage from 1600 until 0800 the next day. The day on which a vaginal plug was seen was designated day 0 of pregnancy.

Chemicals. Penicillin G, streptomycin sulfate, and Trowell's T8 medium were obtained from Gibco Laboratories (Grand Island, NY). DMSO was purchased from Sigma (St. Louis, MO). Coomassie Brilliant Blue G-250 was obtained from Eastman Kodak, Rochester, NY. The metabolizing system consisted of an S-9 fraction (Organon Teknika Corp., Durham, NC), glucose-6-phosphate, and NADPH (Sigma). The protein content of the microsomal fraction was 32.1 mg/ml S-9. PCDEs, PCBs, and 2,3,7,8-TCDD were provided by Dr. Stephen Safe. All other chemicals used in rat embryo culture were from Sigma.

Serum Preparation. Serum used for culture medium was prepared by Hilltop Laboratory, Scottsdale, PA, in accordance with the method of Sanyal and Wiebke (1979). Blood was collected by cardiac puncture from adult male and female rats and immediately centrifuged for 3 min at 1000 x G. The sample was allowed to clot at room temperature for 2 hr and then recentrifuged at 2000 x G. Serum was separated, frozen, and shipped to our laboratory on dry ice. The serum was maintained frozen until heat inactivated (56 °C for 20 min) and aliquoted into culture bottles. Ten milliliters of serum supplemented with penicillin G and streptomycin sulfate (0.6 mg and 0.5 mg, respectively) was placed in 60 ml sterile bottles and sealed with sterile rubber sleeves. Culture bottles were frozen until 30 min prior to use when they were placed in a 37 °C incubator and brought to temperature. For studies of biotransformation, S-9 fraction and co-factors for

monooxygenation were added directly to the media. The co-factors, NADPH and glucose-6-phosphate, were added in final concentrations of 0.5 mM and 5.0 mM, respectively (Faustman-Watts *et al.*, 1983).

Embryo Culture. Embryos were explanted from pregnant females and cultured using procedures described by New (1978) and modified by Sanyal and Wiebke (1979). At 1500 on day 10 of gestation, dams were euthanized using CO_2. Uteri were removed and placed in prewarmed (37 °C) Trowell's T8 medium. Implantation sites were dissected from the uteri, decidua removed, and Reichert's membrane ruptured. Embryos were placed in prewarmed rat serum prepared as above. No more than 5 and no fewer than 4 embryos were placed in each culture bottle. Embryos were untreated or treated with solvent (DMSO, 50 µl/10 ml serum), or one of the chemicals under investigation. Some embryos treated with solvent alone or with 3,3′,4,4′-tetrachlorodiphenyl ether (250 µg/ml) were supplemented with the S-9 metabolizing system (Table 2). Bottles containing the embryos were gassed 2 min with a mixture of 20% O_2, 5% CO_2, and 75% N_2. At +18 hr, embryos were again gassed for 2 min with a gas mixture identical to that used initially. At +30 and +42 hr, the embryos were gassed for 2 min using a mixture of 40% O_2, 5% CO_2, and 55% N_2. Bottles were rolled continuously at 40 rpm for 45 hr. Embryos were maintained at 37 °C throughout the 45 hr culture period.

At +45 hr, embryos were transferred into sterile petri dishes containing sterile normal saline. Viability was determined by examining the embryos under a dissecting microscope for evidence of yolk sac circulation and heart beat. Viable embryos were assessed both qualitatively and quantitatively (yolk sac diameter, crown-rump length,

TABLE 2

Test substances evaluated using post-implantation whole embryo culture.

Group	Test Substance	Concentration	S-9
1	Control		No
2	Solvent Control[a]		No
3	3,3′,4,4′-tetra-CDE	50 µg/ml	No
4	3,3′,4,4′-tetra-CDE	250 µg/ml	No
5	3,3′,4,4′,5-penta-CB	50 µg/ml	No
6	3,3′,4,4′,5-penta-CDE	50 µg/ml	No
7	3,3′,4,4′,5-penta-CDE	250 µg/ml	No
8	2,2′,4,4′,5,5′-hexa-CDE	50 µg/ml	No
9	2,3,3,4,4′,5-hexa-CDE	50 µg/ml	No
10	2,3,7,8-TCDD	200 ng/ml	No
11	2,3,7,8-TCDD	1 µg/ml	No
12	Solvent Control[a]		Yes
13	3,3′,4,4′-tetra-CDE	250 µg/ml	Yes

[a] 0.5% DMSO

somite count, protein, and DNA) for growth and development. Gross morphological examination included inspection for both morphological anomalies and lack of axial rotation. After gross examination, embryos were frozen and maintained at -80 °C until assayed for protein and DNA. Some embryos were fixed in Bouin's fixative in preparation for subsequent histological examination.

Assays. Frozen embryos were placed in 2 ml of phosphate-saline buffer (0.05 M NaH_2PO_4, 2.0 M NaCl, 0.002 M EDTA), pH 7.4. Embryos were homogenized 60 sec at 50% maximum power (Tissumizer, Tekmar) followed by sonication for 35 sec at 20% maximum power (Sonic Dismembrator, Model 300, Fisher). DNA analysis was performed according to the procedures of Labarca and Paigen (1980), using the fluorescent dye, Hoechst 33258. An LS-5 Fluorescence Spectrophotometer (Perkin-Elmer) was used for analysis at an excitation wavelength of 356 nm and an emission wavelength of 468 nm. Protein content was determined in the same sample by the protein-dye binding method of Bradford (1976), using a Beckman DU-65 spectrophotometer at 595 nm wavelength. Tissues and tissue homogenates were maintained on ice throughout the assays.

Histopathology. Representative embryos from 6 different test groups were submitted for histologic evaluation using plastic embedding techniques. These groups were: untreated; 0.5% DMSO; 0.5% DMSO plus S-9 liver fraction; 3,3′,4,4′-tetrachloro-diphenyl ether (250 µg/ml); 3,3′,4,4′-tetrachlorodiphenyl ether (250 µg/ml) plus S-9 liver fraction; and 1 µg/ml 2,3,7,8-TCDD.

Statistical Analysis. Yolk sac diameter, crown-rump length, somite number, total

morphological score, and protein and DNA content were analyzed by Tukey's Studentized Range Test. This test controls the experimentwise error rate, and reduces the probability of falsely declaring significance (Ott, 1988). Data were considered to be statistically significant at the 0.05 level.

Results

Gross examination of embryos revealed no morphological abnormalities following the 45 hr culture period. Of those parameters used to evaluate fetal growth and development, only protein content and crown-rump length showed significant differences ($p < 0.05$) between test groups and controls (Tables 3 and 4). These differences were seen only with 3,3′,4,4′-tetrachlorodiphenyl ether and 3,3′,4,4′,5-pentachlorodiphenyl ether.

There were no significant differences in yolk sac diameter among any of the test groups (Fig. 6). Embryos in the solvent control group had significantly fewer somites than those exposed to rat serum alone (Fig. 7). 3,3′,4,4′-tetrachlorodiphenyl ether (250 µg/ml) plus S-9 caused a significant decrease in crown-rump length when compared to controls (Fig. 8), and embryos in the solvent control plus S-9 group were significantly larger than those exposed to rat serum alone. For total morphological score, embryos exposed to solvent plus S-9 had significantly higher scores than embryos exposed to rat serum alone (Fig. 9). The solvent control plus S-9 group had significantly more DNA (µg/embryo) than the solvent control group and also the group exposed to rat serum alone (Fig. 10). Embryos exposed to 3,3′,4,4′,5-pentachlorodiphenyl ether (250 µg/ml) and 3,3′,4,4′-tetrachlorodiphenyl ether (250 µg/ml) had significantly less protein (µg/embryo)

TABLE 3

Comparison of test compounds on yolk sac diameter, crown-rump length, somite number, and total morphological score.

Treatment	Number of Embryos	Yolk Sac Diameter (mm)[a]	Crown Rump Length (mm)[a]	Number of Somites (Avg)[a]	Morphological Score[a]
Control	21	6.79±0.88	4.30±0.46	28.24±2.07	42.71±3.66
Solvent Control	24	6.54±0.36	3.66±0.47	25.08±1.64	44.50±1.02
3,3′,4,4′-tetra-CDE (50 µg/ml)	4	6.93±0.75	4.36±0.36	25.25±1.50	46.00±0.82
3,3′,4,4′-tetra-CDE (250 µg/ml)	15	6.61±0.70	4.23±0.27	27.80±2.43	47.13±2.17
3,3′,4,4′,5-penta-CB (50 µg/ml)	5	6.13±1.35	4.03±0.62	25.80±3.11	46.40±2.51
3,3′,4,4′,5-penta-CDE (50 µg/ml)	5	6.22±1.11	4.37±1.02	27.80±4.60	42.40±4.22
3,3′,4,4′,5-penta-CDE (50 µg/ml)	9	6.53±1.07	3.50±0.83	26.22±2.54	40.89±3.06
2,2′,4,4′,5,5′-hexa-CDE (50 µg/ml)	5	6.85±0.53	4.24±0.49	28.40±1.52	45.40±0.89
2,3,3′,4,4′,5-hexa-CDE (50 µg/ml)	5	7.06±0.72	3.57±0.59	27.00±3.00	46.00±0.00
2,3,7,8-TCDD (200 ng/ml)	5	6.39±0.77	3.78±0.34	25.80±2.86	44.80±5.12
2,3,7,8-TCDD (1 µg/ml)	6	6.02±0.62	3.63±0.41	23.67±3.01	43.00±4.69
Solvent Control + S-9	9	6.77±0.54	5.15±0.41	28.22±0.67	47.11±1.83
3,3′,4,4′-tetra-CDE (250 µg/ml) + S-9	15	6.24±0.98	4.13±0.56[b]	27.60±3.66	46.87±3.00

[a] Values are means ± standard deviation

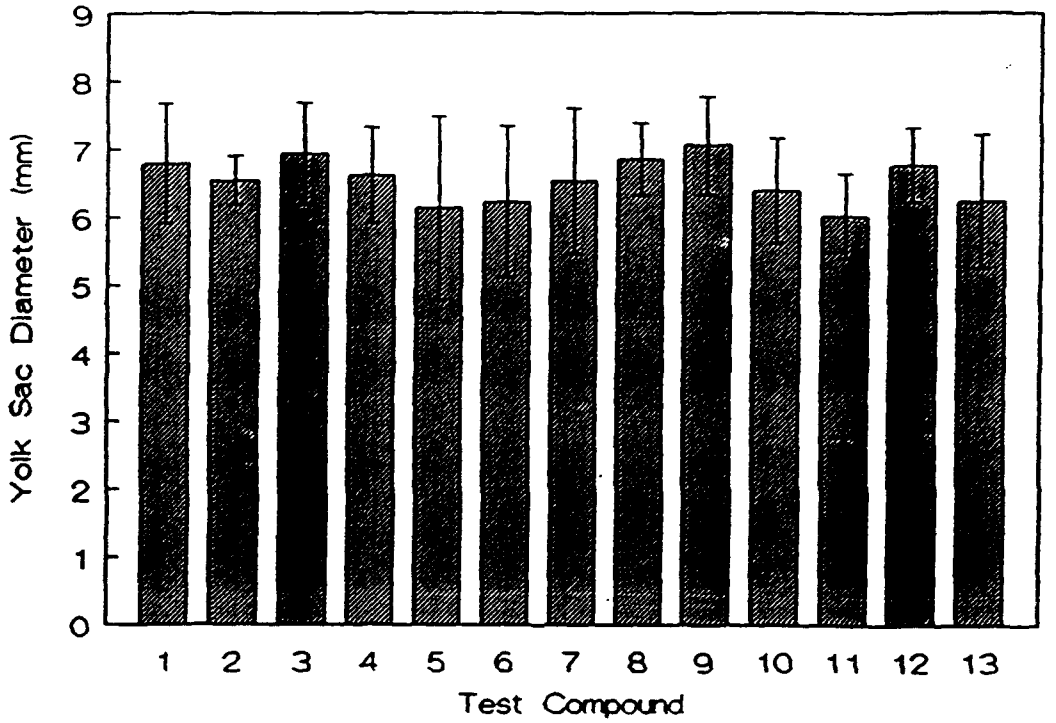
[b] Significantly different from corresponding controls ($p<0.05$)

TABLE 4

Comparison of test substances on protein and DNA content.

Treatment	Number of Embryos	DNA (µg/embryo)[a]	Protein (µg/embryo)[a]
Control	6	44.17±3.76	282.19±19.04
Solvent Control	12	46.17±10.55	242.84±64.03
3,3′,4,4′-tetra-CDE (50 µg/ml)	4	59.50±6.56	259.82±52.97
3,3′,4,4′-tetra-CDE (250 µg/ml)	5	49.0±3.83	122.89±13.35[b]
3,3′,4,4′,5-penta-CB (50 µg/ml)	5	53.80±18.67	314.62±85.24
3,3′,4,4′,5-penta-CDE (50 µg/ml)	5	59.00±14.83	274.18±88.18
3,3′,4,4′,5-penta-CDE (50 µg/ml)	9	33.78±9.82	136.74±36.83[b]
2,2′,4,4′,5,5′-hexa-CDE (50 µg/ml)	5	59.00±18.11	245.85±63.28
2,3,3′,4,4′,5-hexa-CDE (50 µg/ml)	5	48.00±9.90	228.91±44.66
2,3,7,8-TCDD (200 ng/ml)	4	55.50±19.69	268.98±106.30
2,3,7,8-TCDD (1 µg/ml)	6	57.17±12.40	275.48±64.32
Solvent Control + S-9	3	75.67±7.57	183.29±24.21
3,3′,4,4′-tetra-CDE (250 µg/ml) + S-9	10	53.50±15.57	160.39±69.08

[a] Values are means ± standard deviation
[b] Significantly different from corresponding controls ($p<0.05$)

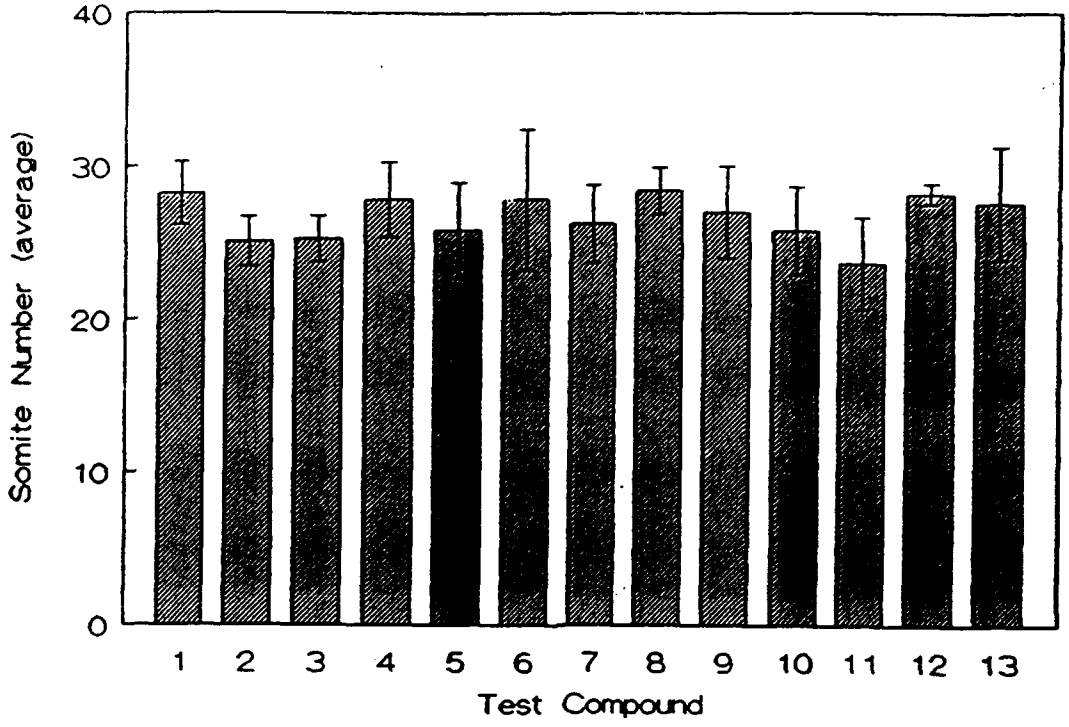


LEGEND

1 - control
2 - solvent control
3 - 3,3′,4,4′-TCDE (50 µg/ml)
4 - 3,3′,4,4′-TCDE (250 µg/ml)
5 - 3,3′,4,4′,5-PCB (50 µg/ml)
6 - 3,3′,4,4′,5-PCDE (50 µg/ml)
7 - 3,3′,4,4′,5-PCDE (250 µg/ml)
8 - 2,2′,4,4′,5,5′-HCDE (50 µg/ml)
9 - 2,3,3′,4,4′,5-HCDE (50 µg/ml)
10 - 2,3,7,8-TCDD (200 ng/ml)
11 - 2,3,7,8-TCDD (1 µg/ml)
12 - solvent control plus S-9
13 - 3,3′,4,4′-TCDE (250 µg/ml) plus S-9

* Significantly different from control values ($p < 0.05$).

FIG. 6. Comparison of yolk sac diameters in rat embryos exposed to various test compounds.

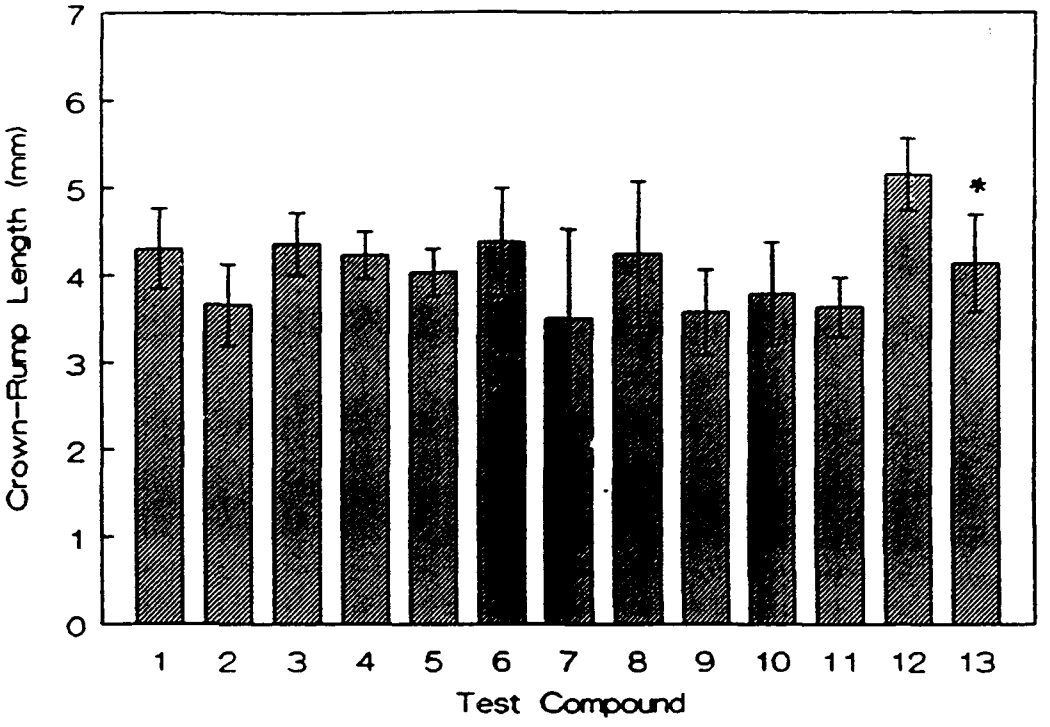


LEGEND

1 - control
2 - solvent control
3 - 3,3′,4,4′-TCDE (50 µg/ml)
4 - 3,3′,4,4′-TCDE (250 µg/ml)
5 - 3,3′,4,4′,5-PCB (50 µg/ml)
6 - 3,3′,4,4′,5-PCDE (50 µg/ml)
7 - 3,3′,4,4′,5-PCDE (250 µg/ml)
8 - 2,2′,4,4′,5,5′-HCDE (50 µg/ml)
9 - 2,3,3′,4,4′,5-HCDE (50 µg/ml)
10 - 2,3,7,8-TCDD (200 ng/ml)
11 - 2,3,7,8-TCDD (1 µg/ml)
12 - solvent control plus S-9
13 - 3,3′,4,4′-TCDE (250 µg/ml) plus S-9

* Significantly different from control values ($p < 0.05$).

FIG. 7. Comparison of number of somites in rat embryos exposed to various test compounds.

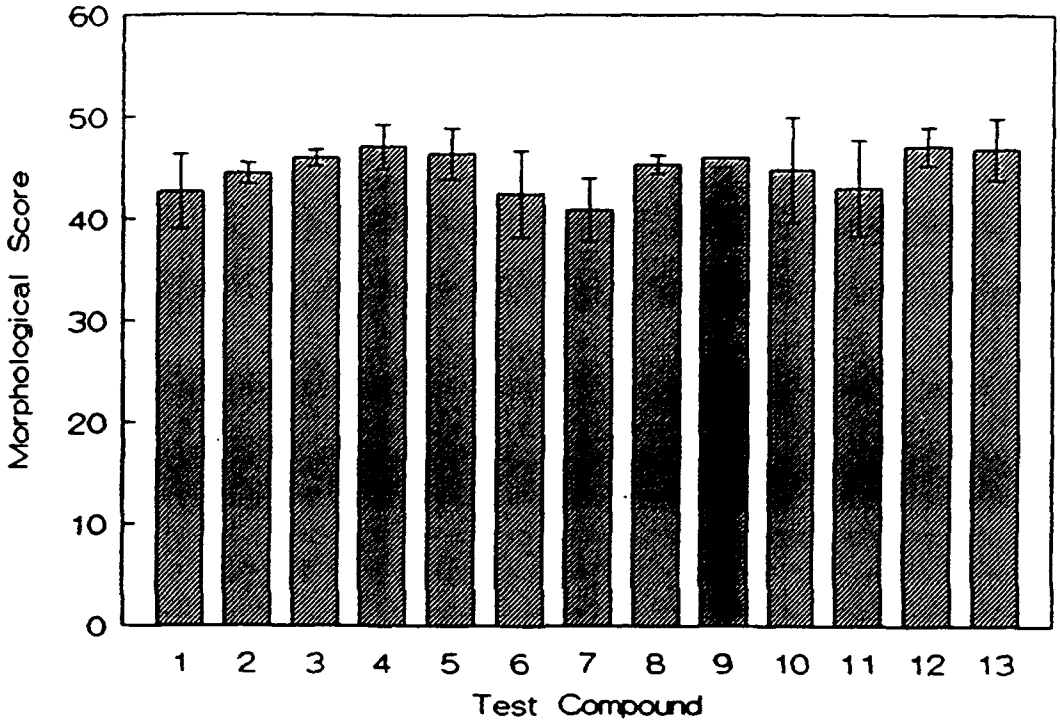


LEGEND

1 - control
2 - solvent control
3 - 3,3′,4,4′-TCDE (50 µg/ml)
4 - 3,3′,4,4′-TCDE (250 µg/ml)
5 - 3,3′,4,4′,5-PCB (50 µg/ml)
6 - 3,3′,4,4′,5-PCDE (50 µg/ml)
7 - 3,3′,4,4′,5-PCDE (250 µg/ml)
8 - 2,2′,4,4′,5,5′-HCDE (50 µg/ml)
9 - 2,3,3′,4,4′,5-HCDE (50 µg/ml)
10 - 2,3,7,8-TCDD (200 ng/ml)
11 - 2,3,7,8-TCDD (1 µg/ml)
12 - solvent control plus S-9
13 - 3,3′,4,4′-TCDE (250 µg/ml) plus S-9

* Significantly different from control values ($p < 0.05$).

FIG. 8. Comparison of crown-rump lengths of rat embryos exposed to various test compounds.

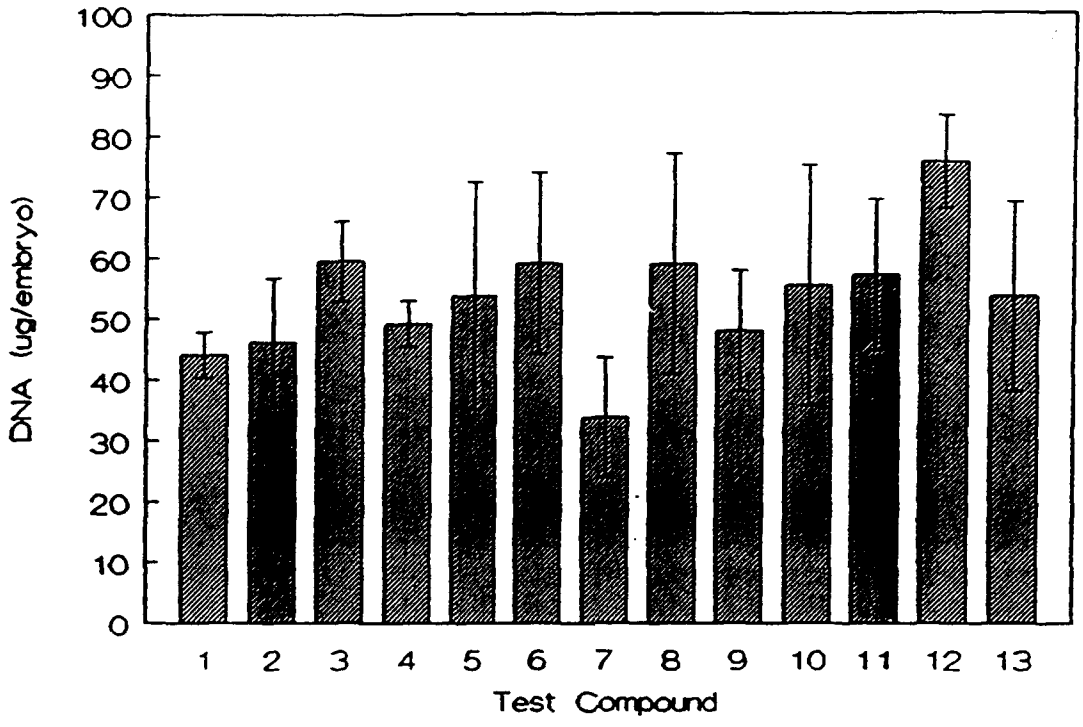


LEGEND

1 - control
2 - solvent control
3 - 3,3′,4,4′-TCDE (50 µg/ml)
4 - 3,3′,4,4′-TCDE (250 µg/ml)
5 - 3,3′,4,4′,5-PCB (50 µg/ml)
6 - 3,3′,4,4′,5-PCDE (50 µg/ml)
7 - 3,3′,4,4′,5-PCDE (250 µg/ml)
8 - 2,2′,4,4′,5,5′-HCDE (50 µg/ml)
9 - 2,3,3′,4,4′,5-HCDE (50 µg/ml)
10 - 2,3,7,8-TCDD (200 ng/ml)
11 - 2,3,7,8-TCDD (1 µg/ml)
12 - solvent control plus S-9
13 - 3,3′,4,4′-TCDE (250 µg/ml) plus S-9

* Significantly different from control values ($p < 0.05$).

FIG. 9. Comparison of total morphological score of rat embryos exposed to various test compounds.



LEGEND

1 - control
2 - solvent control
3 - 3,3′,4,4′-TCDE (50 µg/ml)
4 - 3,3′,4,4′-TCDE (250 µg/ml)
5 - 3,3′,4,4′,5-PCB (50 µg/ml)
6 - 3,3′,4,4′,5-PCDE (50 µg/ml)
7 - 3,3′,4,4′,5-PCDE (250 µg/ml)
8 - 2,2′,4,4′,5,5′-HCDE (50 µg/ml)
9 - 2,3,3′,4,4′,5-HCDE (50 µg/ml)
10 - 2,3,7,8-TCDD (200 ng/ml)
11 - 2,3,7,8-TCDD (1 µg/ml)
12 - solvent control plus S-9
13 - 3,3′,4,4′-TCDE (250 µg/ml) plus S-9

* Significantly different from control values ($p < 0.05$).

FIG. 10. Comparison of DNA content in rat embryos exposed to various test compounds.

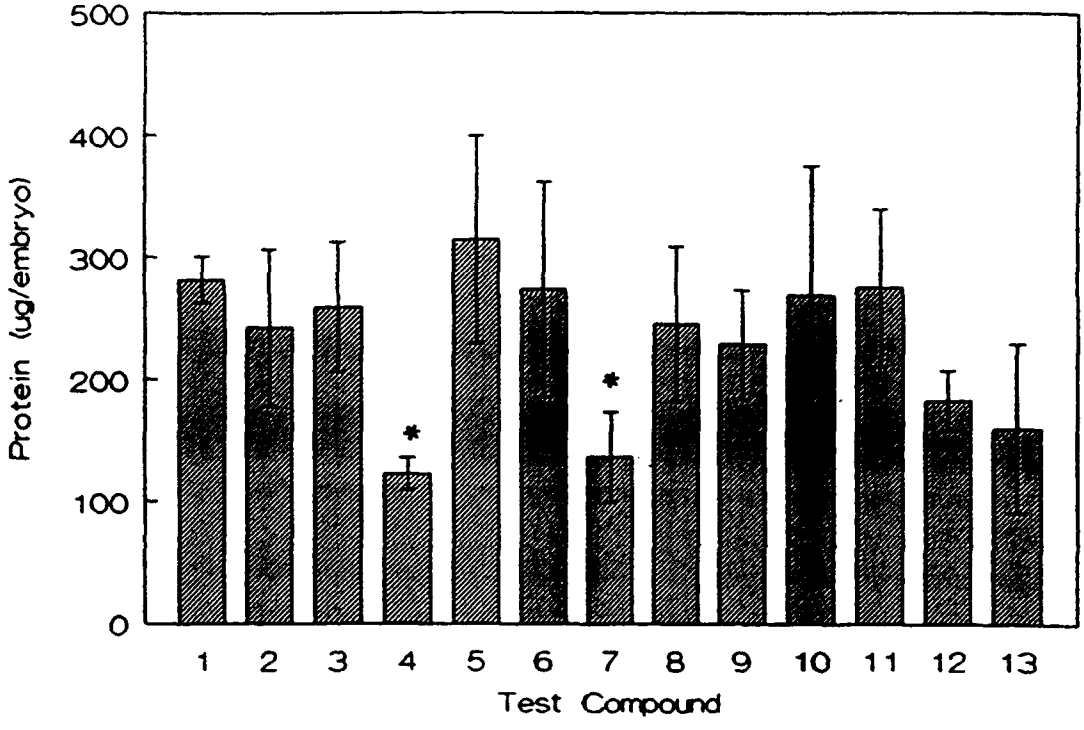
than controls. When 50 µg/ml of these two compounds were used, protein content did not differ significantly from controls (Fig. 11). When embryos were incubated with 3,3′,4,4′-tetrachlorodiphenyl ether (250 µg/ml) plus S-9 and co-factors, protein content did not differ significantly from controls.

In the embryo groups submitted for histological examination, necrosis was evident in some of the embryos but no distinctive pattern, relative to the various treatment groups, could be discerned. Mesenchyme was most severely affected with neuroectoderm and heart relatively spared. The tail region was most severely necrotic.

Discussion

The significant decreases in crown-rump length in embryos exposed to 3,3′,4,4′-tetrachlorodiphenyl ether and protein content in embryos exposed to 3,3′,4,4′-tetrachlorodiphenyl ether and 3,3′,4,4′,5-pentachlorodiphenyl ether are results that warrant further investigation. Both of these PCDEs are coplanar and it was initially suspected that, just as with PCBs, these would be the most active PCDE congeners. However, studies have shown that the addidtion of *ortho* substituents does not necessarily decrease the toxic response of PCDEs as it does with PCBs (Howie *et al.*, 1990). Moreover, Marks *et al.* (1981, 1989), investigated PCBs in outbred albino mice and found that chlorine substituents had to be present in the 3, 3′, 4, and 4′ positions in order for these compounds to exert their teratogenic effects. All rat embryos that showed significantly altered growth or development were substituted with chlorine at the *meta* and *para* positions.

Solubility is a major factor that must be considered when evaluating these results.



LEGEND

1 - control
2 - solvent control
3 - 3,3′,4,4′-TCDE (50 µg/ml)
4 - 3,3′,4,4′-TCDE (250 µg/ml)
5 - 3,3′,4,4′,5-PCB (50 µg/ml)
6 - 3,3′,4,4′,5-PCDE (50 µg/ml)
7 - 3,3′,4,4′,5-PCDE (250 µg/ml)
8 - 2,2′,4,4′,5,5′-HCDE (50 µg/ml)
9 - 2,3,3′,4,4′,5-HCDE (50 µg/ml)
10 - 2,3,7,8-TCDD (200 ng/ml)
11 - 2,3,7,8-TCDD (1 µg/ml)
12 - solvent control plus S-9
13 - 3,3′,4,4′-TCDE (250 µg/ml) plus S-9

* Significantly different from control values ($p < 0.05$).

FIG. 11. Comparison of protein content in rat embryos exposed to various test compounds.

The 3,3′,4,4′-tetrachlorodiphenyl ether, with the fewest chlorine substituents, is the most soluble in aqueous solution or in the culture medium (rat serum). This fact alone may suggest that relatively more of this compound would be distributed to the developing embryo and hence, could more readily exert its toxicity. Additional studies using different solvent systems so that the embryos could be exposed to higher concentrations of the toxins would help clarify this point.

Histological assessment of the effects of test compounds on the developing embryo was not conclusive. There was no evidence to suggest the formation of cleft palate in these rat embryos. Poland and Glover (1980), present evidence which shows that the toxicity of 2,3,7,8-TCDD and related halogenated aromatic hydrocarbons is mediated through the Ah receptor. Results of their studies with inbred strains of mice demonstrate that the susceptibility to cleft palate formation produced by 2,3,7,8-TCDD follows the strain distribution of the Ah locus. The distribution of the Ah locus in developing Sprague-Dawley rat embryo palatal shelves has not been determined. Studies should be conducted, using mouse whole embryo culture, to compare the developmental toxicity of PCDEs in a strain of mice which has a high affinity receptor and is sensitive to enzyme induction (C57BL/6J) with the response in a strain which has a low affinity receptor and is less sensitive to AHH activity (DBA/2J).

Hydronephrosis is one of the most sensitive indicators of 2,3,7,8-TCDD teratogenicity in C57BL/6N mice (Birnbaum *et al.*, 1986). Using the post-implantation whole embryo protocol developed by New (1978), embryonic development is curtailed before the embryonic kidney is developed. Consequently, this parameter could not be

evaluated.

There are many advantages to using post-implantation whole embryo culture as a teratogenic prescreening test. One is that the effects of unmetabolized or parent compounds can be studied by adding these test substances directly to the embryo culture. Comparing these results to those from cultures to which metabolic enzyme systems have been added can help define the role of maternal metabolism in the teratogenic potential of a compound (Fantel, 1982).

On the other hand, one of the major drawbacks to whole embryo culture in screening for teratogens is the relatively brief period of organogenesis during which embryos can be exposed to agents. In the case of the rat embryo, exposed from day 10.5 through day 12.5, many developing systems are not accessible to the test compound and the systems which are available may not include the "target" which would be affected *in vivo* (Fantel, 1982).

Specific defects which develop in whole embryo culture are not necessarily predictive of those which would develop *in vivo*. This discrepancy can have many sources, but is quite likely due to timing differences coupled with repair and resorption processes which are minimal or absent *in vitro* (Fantel, 1982). This fact, however, does not negate the usefulness of whole embryo culture as a teratogenic prescreen.

CHAPTER V

CONCLUSIONS

In vitro assays are currently being developed to study various toxicologic endpoints including teratogenesis. It is important that strict criteria be used in assessing mammalian toxicity models. Goss and Sabourin (1985) propose that the model be sensitive to a chemical, be specific with respect to target tissues, produce similar results upon repetition, be predictive of the toxicologic response in man, and allow extrapolation to man.

The development of *in vitro* toxicology prescreening tests has been of considerable interest to teratologists. Though many *in vitro* tests have been proposed, it is unlikely that any single test will stand alone as a prescreen for chemicals which are potentially teratogenic to humans. It is reasonable to suspect that a teratology prescreening protocol will be developed from a number of standardized *in vitro* tests. We have examined two *in vitro* methods, namely hydra and whole embryo culture assays, for prescreening selected PCDE and and PCB congeners.

The hydra assay provides a unique opportunity to rapidly and inexpensively utilize a lower animal form to study the teratogenic potential of various compounds. We found that one of the major shortcomings of this assay is its inability to respond to compounds with limited water solubility. Progress has been made in this area (Johnson *et al.*, 1988), but additional studies are required to further develop and validate other delivery systems for use with the hydra assay.

Rat whole embryo culture provides the opportunity to study the effects of a toxin on a developing fetus under controlled conditions without the confounding factors of maternal influence. In our studies with embryo culture we found that the halogenated aromatic compounds, at the concentrations tested, had no effect on morphological development when the developing rat embryo was exposed to the compounds, *in vitro*, from days 10.5 through 12.5.

Mammalian whole embryo culture systems are now widely used and have proved useful in many studies of normal and abnormal development. The main advantages of these systems are that they allow precise control of experimental conditions and can often provide information which are not readily obtained from *in vivo* studies; the main disadvantage is the rather short period of embryonic development that can be supported in culture. The possibility of using whole embryo culture systems for screening new teratogenic agents remains controversial, but there are indications that the systems may have a great deal of potential in this area (New, 1990).

For chemicals where a significant risk of human exposure can occur, it is imperative that a single prescreen not be used as the sole means for evaluating toxicity. *In vitro* assays are not meant to replace animal testing, especially for chemicals to which humans are likely to be exposed. They do provide, however, a valuable means for rapid detection and prioritization of developmental toxins.

Research is currently underway to develop and standardize a series of assays, which elicit responses by different mechanisms of action, to prioritize cleanup of oily and wood-preserving hazardous chemical wastes. Previous work in our laboratory has

demonstrated that the hydra assay and post-implantation whole embryo culture are sensitive to some halogenated aromatic compounds such as chlorinated phenols. Our results indicate that these bioassays are not sensitive to PCDEs and PCBs at the concentrations tested, and suggest that these two bioassays, with their differential sensitivities, could be utilized in the hazard and risk assessment of oily and wood-preserving hazardous chemical wastes. Moreover, these assays could also be used to evaluate the effectiveness of clean up procedures at hazardous chemical waste sites.

Wastes that are found at these sites are generally complex mixtures of chemicals. The potential hazards to the environment and to exposed human and animal populations depends upon the toxins present, their concentrations, and their potential for interaction. Extracts from hazardous chemical waste sites containing these heterogenous mixtures, could be evaluated using the hydra assay and whole embryo culture, and provide an opportunity to evaluate antagonistic and/or synergistic interactions when these polyaromatic compounds are found in combination.

The most toxic halogenated aromatic compound is 2,3,7,8-TCDD, and by using *in vivo* and *in vitro* studies, the relative toxicities of individual halogenated aromatics have been determined relative to 2,3,7,8-TCDD (toxic equivalents) (Safe, 1990). The use of toxic equivalents may provide a valuable tool for assessing the risk of halogenated aromatic mixtures, particularly those associated with hazardous chemical waste sites. The ED50 value for the immunotoxicity of coplanar and mono *ortho*- coplanar PCDEs was approximately 10^3 to 10^4 times, respectively, the ED50 value for 2,3,7,8-TCDD immunotoxicity. According to Safe (1990), a toxic equivalency factor value of 0.001

would be a conservative number for both the coplanar and mono*ortho*- coplanar PCDEs.

Additional studies are necessary, but our preliminary results indicate that the hydra and post-implantation whole embryo bioassays can be used as rapid systems for detecting hazardous chemicals, and may provide a valuable adjunct to other analytical methods to detect and rank hazardous chemicals associated with complex mixtures of chemical wastes.

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